Mucosal-associated invariant T (MAIT) cells as cellular adjuvants for cancer immunotherapy

Joshua Lionel Lange

A thesis submitted to the University of Otago in fulfilment of the requirements for the degree of Doctor of Philosophy in Immunology

2020

Abstract

Antigen-presenting cells (APCs), are crucial components of the immune system that are uniquely positioned at the interface between innate and adaptive immunity. In particular, a specialized subset of APCs, dendritic cells (DCs), play a pivotal role in initiating T and B cell responses. This thesis focuses on the initiation of T cell responses, a process that requires DCs to acquire antigens derived from infected or malignant cells and process them for presentation to T cells with appropriate antigen receptors. The DCs also have to receive distinct stimulatory signals in order to increase their expression of chemokines, adhesion molecules, co-stimulatory molecules and cytokines. These phenotypic changes, known as DC activation, facilitate interactions with antigen-specific T cells to promote their proliferation and differentiation into effector cells. Thus, compounds that enhance DC activation can be utilized as vaccine adjuvants; as such, they are highly sought after in the development of novel immunotherapies.

Innate-like T cells express many of the features of conventional T cells but lack diversity in their antigen receptors, and exist poised in a semi-activated state, more like cells of the innate system. Of the different innate-like T cells subsets, CD1d-restricted natural killer T (NKT) cells are known to be potent sources of stimulatory signals that induce activation of DCs. Another innate-like T cell subset, mucosal-associated invariant T (MAIT) cells, have primarily been regarded as anti-bacterial effector T cells because they react to pyrimidines derived from bacterial riboflavin synthesis. However, because they share many phenotypic properties with NKT cells, it is possible that MAIT cells could also play a role in influencing adaptive immune responses by modifying the phenotype of DCs. Indeed, at the start of this thesis, a study was published showing that activated human MAIT cells could induce activation of monocyte-derived DCs *in vitro*. Importantly, MAIT cells are highly abundant in humans and are enriched at mucosal sites, potentially making them useful cells to exploit in vaccine designs that can be clinically translated. Therefore, this thesis sought to determine if agonists that activate MAIT cells could act as adjuvants by facilitating cellular interactions that modulate the functionality of DCs *in vivo* in order to augment priming of antigen-specific T cell responses.

The MAIT cell agonist precursor, 5-amino-6-D-ribitylaminouracil (5-A-RU), forms pyrimidine intermediates with cellular by-products of metabolism that bind to the MHC I-related protein 1 (MR1) which engages and activates MAIT cells. For example, when

5-A-RU condenses with methylglyoxal (MG), the potent agonist 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) is formed. One major challenge in MAIT cell research is that 5-OP-RU is an unstable compound. To circumvent this problem, 5-A-RU was used as it is known to condense with endogenous metabolic by-products to form the agonists intracellularly for *in vitro* or *in vivo* studies. However, in this thesis, it is shown that 5-A-RU is also unstable, being labile to auto-oxidation resulting in the loss of biological activity. To overcome these challenges, a 5-A-RU prodrug was developed that impeded oxidative degradation and conferred enhanced MAIT cell-activating properties both *in vitro* and *in vivo*. Furthermore, this prodrug design also provided a scaffold for chemical conjugation of model antigens to 5-A-RU to facilitate co-localisation of both adjuvant and antigen within the same DC, a mechanism known to enhance T cell priming. In addressing the hypothesis that MAIT cells could help initiate T cells responses, it was initially shown that intravenous administration of 5-A-RU *in vivo* resulted in DC activation, and when co-administered with soluble antigen, resulted in antigen-specific T cell immunity. Moreover, 5-A-RU-peptide conjugates showed enhanced DC functionality compared to 5-A-RU and drove effective anti-tumour activity. However, further analysis of these compounds revealed the presence of an unexpected, unknown microparticle that activated CD1d-restricted NKT cells, with the NKT cells being the source of the cellular adjuvant activity observed; this observation was shared by colleagues with 5-A-RU manufactured from a different laboratory.

Elimination of the confounding NKT cell-activating microparticle by fractionation or filtration revealed that MAIT cells could still facilitate DC activation, but only when the filtered 5-A-RU was combined with MG to form 5-OP-RU immediately before use. Activation of DCs was only readily detected in the lungs and lung-draining lymph nodes, with MAIT activity potentially driving DC migration from the lung tissue to the lymph nodes. Interestingly, administration of antigen with 5-OP-RU formed from filtered 5-A-RU failed to generate antigen-specific T cells. However, co-administration antigen with 5-OP-RU and limited doses of a toll-like receptor (TLR) agonist provided additional stimulatory signals, enhanced DC activation and resulted in MR1-dependent expansion of antigen-specific T cells.

In summary, this thesis presents evidence that MAIT cells can influence innate and adaptive immune responses. However, the activity of MAIT cells alone is insufficient to

initiate T cell responses, as additional signals are required. Nonetheless, this feature of MAIT cell function could be incorporated into vaccine strategies in order to drive antigen-specific T cells to pathogen- or tumour-associated antigens for the development of future immunotherapies.

Acknowledgements

Completing this thesis has been one of the most challenging but rewarding experiences I have ever had to undertake and none of it would have been possible without the continual support from the many people in my life.

Firstly, I would like to thank my supervisor, Prof. Ian Hermans. Over the last four years you have been a constant source of support, knowledge and advice. Thank you for giving me the tools and the freedom to explore my ideas and teaching me how think critically about my research within the wider context of immunology. I have truly valued your mentorship.

To Prof. Brett Delahunt, thank you for the continual support over these last few years and allowing me to undertake a PhD through the University of Otago.

To everyone in the Cancer Immunotherapy Programme, every single one of you has contributed positively to my PhD experience in some way. Thank you to our amazing research officers Kathryn Farrand, Chingwen Tang, Astrid Authier-Hall, Anna Mooney and Phoebe Harmos. None of the work I have done could have been possible without all the work you put in towards maintaining the lab and the technical expertise you provide. Taryn Bingham, thank you for showing me how to be a pro at *in vivo* manipulations. Laura Ferrer-Font, thank you for your help with the design and optimisation of big Aurora panels.

To my Malaghan friends and fellow students, thank you for providing me with unwavering support and friendship. In particular thank you to Anna Mooney, Ellie-May Jarvis, Kirsty Wakelin and Jodie Chandler. The laughs, advice and booze shared over the years has kept me from the brink of insanity.

A very special mention has to go to my PhD twin sister, Olivia Burn. It has been the most amazing pleasure to share this journey with you. Over these last four years I have been able to watch you grow into a confident and amazingly intelligent scientist. I am so excited to see where both of our paths lead us and perhaps one day we may see them overlap again.

This research would not be possible without the financial contributions from the Health Research Council of New Zealand and the University of Otago. I was privileged enough to also receive grants from Research for Life, Cancer Society and the University of Otago which allowed to travel and share my work at international conferences. These experiences were helped shape me as a scientist and allowed to make invaluable connections around the world.

A special mention to the Maurice Capstick Foundation which provided a substantial financial contribution towards my project that allowed me to travel and work at Oxford University for four months. Being able to have this experience was truly priceless and has changed my life for the better – thank you.

A very special thank you to the family of Peter Lapsley for donating to my research project; know that your generosity has given me the ability explore and contribute to the field of cancer immunology with the hopes that one day this work can inform the generation of new therapies in the clinic.

To all my family and friends, thank you for everything you have given to me on this path. To my chosen sisters, I always be grateful for the wild nights and adventures we have shared together. To Mum, Dad and my brother Dylan, thank you for being my biggest cheerleaders and making me see the bigger picture of everything I do.

To Max, I cannot begin to thank you for your support, love and the sacrifices you have made for me on my PhD journey. You have been the calm to my storm through everything. Mahal na mahal kita.

Disclosure statement

The data contained within chapter 3 of this thesis was generated through collaborative efforts and includes material that has been submitted and accepted for publication (See Publications). Chemical synthesis and analysis of the compounds used within this chapter were performed by Dr Regan J. Anderson from the Ferrier Research Institute, Wellington, NZ. Specific contributions are detailed within the text.

With this disclosure, I declare that the content within this thesis is my own work.

Abbreviations

α-GalCer	α-Galactosylceramide
Ac-6FP	Acetyl-6-formylpterin
Ag	Antigen
APC	Antigen presenting cell
BAL	Bronchoalveolar lavage
BCR	B cell receptor
BCG	Bacille de Calmette et Guérin
BMDC	Bone marrow derived DC
BST-2	Bone marrow stromal Ag 2
CD	Cluster of differentiation
cDC	Conventional DC
CDP	Common DC progenitor
CFSE	Carboxyfluorescein succinimidyl ester
Clec9A	C-type lectin domain family 9 member A
CLIP	Class II-associated invariant chain peptide
CMV	Cytomegalovirus
CTL	Cytotoxic lymphocyte
CTO	CellTracker™ Orange
CXCR	C-X-C chemokine receptor
CyTOF	Mass cytometry
DC	Dendritic cell
DN	Double negative
DTR	Diptheria toxin receptor
EDS	Energy Dispersive X-ray spectroscopy
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
eYFP	Enhanced yellow fluorescent protein
Exo-BCN	Bicyclo[6.1.0]non-4-yne
FACS	Fluorescence-activated cell sorting
G-CSF	Granulocyte colony stimulating factor
GPI	Glycosylphosohatidylinositol
HIV	Human immunodeficiency virus

HPV	Human papilloma virus
HR	Hazard ratio
ICOS	Inducible T cell costimulator
Ii	Invariant chain
IFN	Interferon
IFNAR	IFN α/β receptor
IL	Interleukin
MFI	Mean fluorescence index
LAG3	Lymphocyte activation gene 3 protein
LCMS	Liquid chromatography mass spectrometry
LCMV	Lymphocytic choriomeningitis virus
LPS	Lipopolysaccharide
MAIT	Mucosal-associated invariant T
MFI	Median fluorescence index
MG	Methylgloxal
MHC	Major histocompatibility complex
moDC	Monocyte-derived DC
MR1	MHC-I related gene 1
MyD88	Myeloid differentiation primary response 88
NETs	Neutrophil extracellular traps
NFκB	Nuclear factor kappa light chain enhancer of activated B cells
NKT	Natural killer T
NP	Nanoparticles
pDC	Plasmacytoid DC
PBS	Phosphate-buffered saline
Pd	Palladium
PD-1	Programmed cell death protein 1
PLZD	promyelocytic leukaemia zinc finger protein
PRR	Pattern recognition receptor
RORγt	Retinoic acid-related orphan receptor gamma t
SEM	Scanning electron microscopy
SPAAC	Strain-promoted alkyne-azide cyclo-addition
SPF	Specific pathogen free
STAT	Signal transducer and activator of transcription

TAP	Transporter associated with antigen processing
Tbet	Transcription box protein expressed in T cells
TCR	T cell receptor
TEM	Transmission electron microscopy
T_{fh}	T follilcular helper
Th	T helper
TIL	Tumour infiltrating lymphocyte
TNF	Tumour necrosis factor
TLR	Toll-like receptor
TRAF	TNF receptor associated factor
TRAJ	TCRα joining region
TRIF	TIR domain containing adapter inducing interferon β
WT	Wild-type
XCR1	XC Motif Chemokine Receptor 1
VC-PAB	Valine-citrulline-p-aminobenzyl carbamate
5-A-RU	5-amino-6-D-ribitylaminouracil
5-MOP-RU	5-(1-methyl-2-oxopropylideneamino)-6-D-ribitylaminouracil
5-OE-RU	5-(2-oxoethylideneamino)-6-D-ribitylaminouracil
5-OP-RU	5-(2-oxopropylideneamino)-6-D-ribitylaminouracil
6-FP	6-formylpterin

Table of contents

List of Figures

Chapter 1:General Introduction

The generation of potent immune responses to pathogens or malignant tissues requires a concerted effort between numerous immune cell-types within the host through distinct molecular signalling pathways. To initiate this process, host immune cells specialised in sampling the local environment must acquire foreign or mutationally altered molecules known as antigens, and process them into structures that can be recognised and targeted by the other cells of the immune system[1]. These initiating cells, known as antigen presenting cells (APCs), are part of the innate immune system, and conduct their activities rapidly after they recognise molecules (or "patterns") commonly associated with infection or cellular distress *via* innate pattern-recognition receptors (PRRs)[2,3]. Importantly, APCs are vital in driving the activation and differentiation of a specialised subset of lymphocytes, called T cells, which are the major topic of this thesis. The importance of T cells lies in their ability to clonally expand and penetrate infected or malignant tissues in large numbers in order to selectively eliminate cells that express the foreign or mutationally altered molecules they recognise. This high degree of selectivity to antigen, which takes several days to develop provides the precision required to eliminate infectious tissue with minimal danger to self-tissues (although this risk is not entirely removed) and is the basis of adaptive immunity[4–6]. Adaptive immunity also involves lymphocytes called B cells that release antibodies that can target the three-dimensional structure of pathogen-derived antigens[7]. Though both T and B cell responses are critical determinants in sterilizing immunity in their own right, the focus of this thesis will focus on the development of T cell responses.

Due to their pivotal role in T cell-based immunity, the molecular mechanisms that govern APC-T cell cross-talk have been of particular interest to immunologists, especially for the development of novel vaccine strategies and cancer immunotherapies. It has been well established that the presence of microbial or tumour-derived antigen alone is generally insufficient to drive sterilising or protective immunity[8–10]. Successful generation of antigen-specific immune responses requires auxiliary signals that drive the activation of the APC *vi*a PRRs. Where substances that stimulate this process have been added to vaccines to increase activity, the compounds involved are known as immune adjuvants. Adjuvants can also include compounds that help in the uptake and processing of antigens. The term adjuvant, in some cases, has become adapted to refer to the process of activating APCs, particularly when referring to third party cells that can be stimulated to perform this function, which is a primary topic of this thesis. These cells are sometimes, therefore,

referred to as “cellular adjuvants”, or as performing an “adjuvant function”. In microbial infection, common microbial signatures such as cellular structures like flagella, or nucleic acid structures such as unmethylated DNA or double-stranded RNA, provide these adjuvant signals and are recognised by APCs via PRRs[2,3,11]. Of these receptors, Toll-like receptors (TLRs) are perhaps the best-described. Recognition induces a signal cascade resulting in the activation of APCs, allowing them to effectively present antigen to T cells in a stimulatory context, prompting T cell activation. Use of synthetic TLR agonists has therefore been an attractive approach in vaccine development, especially for cancer immunotherapy as cancer cells do not naturally produce these APC maturing signals.

However, APCs can also be matured through contact-dependent cellular signalling with other cells. The most well-recognised cell-types capable of this function are antigen-specific $CD4^+$ T cells, ultimately providing a positive feedback loop (or “help”) that co-operates with pattern-recognition to improve APC function[12–15]. During antigen presentation to $CD4^+$ or $CD8^+$ T cells, processed peptide fragments are presented *via* major histocompatibility complex (MHC)-II or MHC-I molecules, respectively, on the APC surface to antigen receptors on the T cells (referred to as T cell receptors; TCR). In particular, during interactions with $CD4^+$ T cells, APC maturation is enhanced through the provision of the ligand for CD40, a co-stimulatory molecule on APCs (whose expression, in turn, can be improved in response to pattern-recognition)[15–17]. This co-stimulation is a critical step in initiating robust T cell responses, particularly those involving $CD8^+$ T cells, which differentiate into cytotoxic T lymphocytes (CTL).

Several animal models have shown that another T cell subtype, defined by their largely invariant TCR, referred to as type I NKT cells, are also poised to provide these ‘helper’ signals to APCs[18–20]. *In vivo* research utilising a synthetic agonist for NKT cells, α-galactosylceramide (α-GalCer) has shown that NKT cells potently enhance the maturation of APCs through CD40/CD40L signalling. Due to this, α-GalCer has been used as a vaccine adjuvant, where co-administration with peptide or protein antigens induces the expansion of antigen-specific T cells. Perhaps surprisingly, this powerful response does not require any additional pattern recognition, although responses can be enhanced further if this is also supplied[21]. Specifically, the use of α-GalCer has shown promising results in cancer immunotherapy in various *in vivo* models, and use of α-GalCer as an adjuvant in the clinical setting is currently under investigation.

Unfortunately, a clear limitation of NKT cell-based vaccine strategies is a significant difference in the frequency of these cells in humans compared to mice. Mice NKT cells outnumber those found in humans by up to 100-fold[22]; however, it is currently unknown whether this substantial reduction in numbers will hamper the quality of signalling provided to APCs within a human setting. Recently another T cell-subtype, MAIT cells, has been shown to not only be enriched in humans but also to display many functional similarities to the NKT cell phenotype[23–25]. Early reports suggest that MAIT cells may also be able to influence adaptive immune responses[26–28]. Critically, a recent publication has indicated that MAIT cells have the capacity to induce the maturation of APCs through CD40/CD40L when activated, in a similar manner to NKT cells[29]. However, the question remains whether MAIT cell-mediated APC maturation is sufficient to drive subsequent antigen-specific T cell responses. Therefore, this thesis aims to investigate the ability of MAIT cells to drive APC maturation *in vivo* to prime antigen-specific T cell responses. This introduction will review the background literature describing the generation of adaptive T cell responses as well as a summarise the evidence that MAIT cells may be able to augment this function for the development of novel immunotherapies.

1.1 Initiation of the adaptive immune responses

1.1.1 Antigen processing and presentation

The innate immune system consists of a plethora of cells that primarily act as an early barrier against infectious agents. Recognition of invading pathogens by the innate immune system is mediated by conserved germline-encoded PRRs which sense conserved microbial motifs that rapidly trigger a variety of anti-microbial immune responses[30]. The concerted action between cells of the innate immune system is efficient and fast-acting, however, lacks precise specificity. Another key feature of the innate immune response is the ability to provoke the activation of adaptive immune cells, i.e. T and B cells. These cells have randomly generated receptors specific for a given antigen and begin a process of clonal selection upon recognition of their cognate antigen. During this process, antigen recognition drives the selective expansion of reactive T or B cells clones, therefore providing pathogen-specific immunity.

The focus of this thesis will be on the T cell-mediated arm of adaptive immunity which recognises antigen through randomly generated TCRs. To initiate T cell responses to a given pathogen, antigens must be loaded onto MHC molecules within APCs[31]. Once processed by an APC, these antigen (Ag)-MHC complexes are expressed on the cell surface where they can be recognised by T cells through their TCR to drive their activation. Two classical MHC molecules, MHC-I and MHC-II, are responsible for the presentation of short peptide fragments to engage with the TCR of $CD8^+$ and $CD4^+$ T cells, respectively. When T cells recognise these Ag-MHC complexes on APCs that have been activated by exposure to pathogen-derived (or other "danger-associated") molecular structures, they undergo clonal proliferation and distinct phenotypic changes ultimately resulting in the capacity to clear pathogens or eliminate malignant tissue.

1.1.1.1 Presentation of antigen on MHC class II molecules

Initiation of the adaptive immune response first begins with recognition and uptake of extracellular bodies or proteins into APCs. Cell-associated antigens, such as whole bacteria or apoptotic bodies, are taken up into endocytic vesicles in a process known as phagocytosis[32,33]. These 'phagosomes' undergo a series of maturation events that includes fusion with acidified lysosomes. These lysosomes, containing host proteases, enzymatically digest proteins into antigenic peptide fragments[34]. Exocytic vesicles

derived from the endoplasmic reticulum (ER) contain MHC-II bound to an invariant chain (Ii), which inhibits premature binding of peptides to MHC-II. Exocytic vesicles then migrate through the trans-Golgi network and fuse with antigen containing phagosomes to form phagolysosomes[32]. Fusion with these exocytic vesicles brings processed antigen in contact with MHC-II molecules. Proteases such as Cathepsin S within the newly formed phagolysosome cleave Ii within the antigenic MHC-II binding groove, leaving behind a low-affinity Ii derived self-peptide (CLIP)[35,36]. The acidified environment within the phagolysosome now catalyzes the exchange of CLIP with the exogenously-derived antigenic peptides forming mature MHC-II-peptide complexes[37]. Finally, these complexes are transported to the cell surface where they can be recognised by cognate T-cell receptor (TCRs) on $CD4^+$ T cells initiating an adaptive immune response to extracellular pathogens.

1.1.1.2 Presentation of antigen on MHC class I molecules

For the host immune system to sense intracellular infection or formation of aberrant cells, cytosolic proteins are continually degraded by a collection of enzymes known as the proteasome[38]. Processed peptide fragments derived from the proteasome are then chaperoned via transporter associated with antigen processing (TAP) molecules into the lumen of the ER and loaded onto newly synthesised MHC-I molecules. Newly formed Ag-MHC-I complexes then translocate to the cell surface where they can engage with $CD8^+$ T cells *via* a cognate T cell TCR interaction. Almost all nucleated cells possess the ability to present these endogenously-derived antigens on MHC-I[39]. Thus, providing an essential mechanism by which neoplastic cells that express aberrant proteins (such as in cancer), or cells infected by intracellular pathogens can become tagged for destruction by $CD8^+$ T cells.

MHC-I also plays a crucial role in the presentation of exogenous antigens derived from phagocytosed or endocytosed material in a process known as cross-presentation. Although many studies have described various methods in which exogenous antigens can become associated with MHC-I, in general, there are two main pathways of cross-presentation: the cytosolic pathway or the vacuolar pathway. In the cytosolic pathway, a proportion of internalised material can ‘escape’ endosomes into the cytosol to be processed by the proteasome and amino- or carboxyl-terminal peptidases[40,41]. Antigen escape is enhanced in some DC subtypes, namely cDC-1s (discussed later), which in part

could be attributed to elevated levels of molecules such as NOX2 that prohibits rapid acidification of endosomes allowing prolonged antigen stability[42,43]. From here, the antigen enters the canonical pathway for MHC-I presentation *via* association with TAP followed by loading of MHC-I within the ER. In 1995, Kovacsovic *et al.* showed that cross-presentation could be abolished by blocking the transport of ER proteins to the Golgi with Brefeldin A or through the mutation of the TAP1/TAP2 genes[44]. However, subsequent studies showed cross-presentation could be achieved independent of both ER transport and TAP, suggesting a mechanism by which cross-presentation could occur outside the cytosolic pathway[45,46]. The model, termed the vacuolar pathway, was proposed after cross-presentation was demonstrated in a proteasome-independent but endosomal acidification-dependent manner[47,48]. In this way, in a similar manner to MHC-II presentation, the endocytosed antigen is instead processed by endosomal proteases. Subsequently, antigenic peptides are loaded onto endosomal-associated MHC-I molecules. This was demonstrated *in vivo* in mice that lacked the endosomal cysteine protease, Cathepsins S, which showed reduced antigen generation when administered cell-associated antigen[47].

1.1.2 Dendritic cells

The ability of cells to capture and process antigen onto MHC molecules efficiently is critical in the generation of robust adaptive immunity. While a number of cells possess the capacity to capture and present antigens, including B cells and macrophages, DCs are considered the most proficient due to their heightened ability to uptake antigen and high expression of MHC and T cell co-stimulatory markers compared to other APCs[1,49–51]. Dendritic cells originate from the bone marrow from a common dendritic cell precursor (CDP) and are further delineated into two sub-categories; conventional DCs (cDC) or plasmacytoid DCs. Dendritic cells reside ubiquitously throughout the body within both secondary lymphoid organs and in peripheral tissues such as the skin, liver and mucosa[52–56]. Additionally, under inflammatory conditions, Ly6C^{hi} monocytes can also differentiate *in situ* into monocyte-derived DCs that migrate to secondary lymphoid organs and gain an increased capacity to present antigen[57]. As this thesis focuses on *in vivo* murine studies, the following section on DC subtypes will focus on literature from murine studies, particularly in reference to phenotypic surface markers.

1.1.2.1 Conventional DCs

Conventional DCs are a diverse set of cells that act as the sentinels of the immune system, dedicated to sensing and sampling their local environment. Conventional DCs can be roughly divided into two subsets: resident cDC and migratory cDC. Resident cDCs, primarily populate secondary lymphoid organs and exhibit an 'immature' phenotype of low expression of MHC and T cell co-stimulatory markers: CD40, CD80 and CD86[58]. In contrast, migratory cDCs are located at peripheral tissues and constitutively circulate through the lymphatic system into draining lymph nodes[59]. During their circulation, even in the absence of microbes and microbial signalling, these DCs obtain increased levels of T cell co-stimulatory markers, enhancing engagement with naïve T cells within the lymph node[60]. This function highlights an important role for migratory DCs in the sampling of peripheral 'self' antigen to induce T cell tolerance – the process of inactivating or deleting autoreactive T cells. Conventional DCs can also be further delineated based on their ontogeny which dictates distinct functional differences; these are cDC-1 and cDC-2.

The development of the cDC-1 population relies on the transcription factors Batf-3 and IRF8[61,62]. Both lymphoid organ-resident and migratory populations of Batf-3 dependent cDC-1s exist, identifiable by their expression of CD8α and CD103, respectively. Importantly, cDC-1s have a critical role in cross-presentation and the stimulation of effector $CD8^+$ T cells. While both cDC-1 and cDC-2 subsets show an equal capacity to capture antigen and present them on MHC-I and MHC-II molecules; it is generally regarded that cDC-1 are the most effective at cross-priming $CD8^+$ T cell responses [63,64]. This was demonstrated *in vivo,* where mice lacking Batf-3 lose their ability to cross-present soluble or cell-derived antigens[65]. Their role as efficient cross presenters is also emphasised by their increased expression of molecules associated with antigen capture, *i.e.*, c-type lectins DEC205, CD207 and Clec9A[66–68], as well as higher expression of proteins involved in cross-presentation such as Tap and Sec61b[69,70]. Furthermore, constitutive expression of the chemokine receptor, XCR1, which binds to its cognate ligand XCL1 expressed by $CD8^+$ T cells, is thought to facilitate the development of effector $CD8^+$ T cells[71].

In contrast to the homogenous subset of cDC-1s, cDC-2s are a heterogenous population of DCs that differ in their transcription factor expression and development but can also be divided into resident and migratory populations. Several different transcription factors

are reported to be involved in the development of the cDC-2 subset including Notch2, KLF4 and IRF4[70,72,73]. Notably IRF4 expression enhances MHC-II expression within the cDC-2 subset and drives migration from the periphery to secondary lymphoid organs[74,75]. In line with this, cDC-2s have been emphasised as key drivers in the activation and differentiation of $CD4^+$ T cells[76,77].

1.1.2.2 Monocyte-derived DCs

Monocyte-derived DCs (moDC) represent a highly plastic subtype of cells that share many functional similarities with the broader cDC subset. Rather than being a *bona fide* DC subtype of their own, under inflammatory settings, monocytes acquire functional capabilities reminiscent of cDC as well as increased levels of CD11c, MHC-II and T cell co-stimulatory markers, but can be differentiated from cDC based on the expression of the monocyte marker CD64[78,79]. *In vivo* studies demonstrate that during bacterial infection with pathogens such as *Leishmania* and *Citrobacter rodentium*, circulating monocytes are recruited to the site of infection where the inflammatory stimulus drives differentiation into moDCs[57,80]. Once differentiated, moDCs become active producers of the pro-inflammatory cytokine IL-12, supporting $CD4^+$ T cells priming[81].

1.1.2.3 Plasmacytoid DCs

Plasmacytoid DCs (pDC) are a distinctive subset of DCs found highly localised at steady state within secondary lymphoid organs,[82–84] and show some capability to home to peripheral sites during inflammation[85,86]. As with other DC populations, pDCs are characterised by their expression of CD11c[83]. Although, in contrast to the other DC subsets, pDCs also resemble B cells in that they express high levels of the CD45 isoform, B220 (CD45R), as well as expression of the sialic acid-binding immunoglobulin-type lectin (Siglec)-H. The primary role of pDCs is in recognition of viral-derived motifs, such as unmethylated CpG dinucleotides or double-stranded RNA, on intracellular sensing receptors - TLR7 and TLR9, respectively[87,88]. Upon activation, pDCs are avid producers of type-I interferons, IFNα and IFNβ, due to their constitutive expression of the transcription factor IRF-7[89]. Activated pDCs also undergo distinct morphological and transcriptomic changes that are reminiscent of a matured cDC phenotype, upregulating MHC-I, MHC-II and T cell co-stimulation molecules CD40, CD80, and CD86[90,91]. *In vitro* and *in vivo* studies addressing the ability of pDCs to present antigen have revealed that whilst activated pDCs are able to process and present endogenous antigen on MHC-

I or II, they lack the ability to effectively cross-present exogenous antigens onto MHC-I molecules[92–94].

1.1.3 DC maturation

At the cross-section between innate and adaptive immunity, DCs must undergo necessary phenotypic and morphological alterations to transition from an immature antigen-capturing cell to a mature antigen-presenting cell, which allows them to prime naïve T cells. At the immature stage, DCs have high endocytic capacity enabling them to survey and capture antigens[95]. This stage is characterised by low expression of antigen-presenting molecules, MHC-I and MHC-II as well as low levels of T cell co-stimulatory markers, CD86/80 and CD40. Therefore, immature DCs are poor stimulators of T cell responses. During maturation, DCs upregulate these co-stimulatory markers and also upregulate chemokines and adhesion molecules which facilitates recruitment and cell contact between naïve T cells and DCs[96–98]. However, merely acquiring antigen in isolation does not induce maturation of DCs. In fact, in steady-state, presentation of antigen to naïve T cells by immature DCs results in the induction of tolerance or generation of anti-inflammatory regulatory T cells[8–10]. Thus, to bypass promotion of tolerance, immature DCs must receive accessory signals in order to upregulate co-stimulatory markers and expression of cytokines resulting in activation of naïve T cells.

During the course of an infection, germline-encoded PRRs, which include TLRs, recognise extra- and intra-cellular microbial-based signatures. TLRs such as TLR1, TLR2, TLR4, TLR5, and TLR6 are expressed on the cell surface and recognise extracellular microbial motifs such as lipopeptides, flagellin and lipopolysaccharides (reviewed here[99]). Bacterial-derived unmethylated DNA can also be recognised intracellularly by TLR9, or virally infected cells can sense single or double-stranded RNA on TLR3 and TLR7, respectively. Recognition of these signatures are not only key in the initiation of innate immunity by promoting inflammation, but also play a critical role in DC maturation[11,99]. TLR ligation in DCs results in a signal cascade mediated through adaptor molecules such as TIR domain-containing adapter inducing interferon β (TRIF) and Myeloid differentiation primary response 88 (MyD88), that results in expression of transcription factors such as Nuclear factor kappa light chain enhancer of activated B cells (NFκB) and various Interferon regulatory factors (IRFs)[100,101]. These activated DCs acquire the ability to migrate to local lymph nodes and

upregulate MHC molecules, which enhances DC-T cell interactions, as well as expression of co-stimulatory markers CD86/80, which bind to CD28 on T cells. Furthermore, activated DCs can now produce pro-inflammatory cytokines, such as IL-12, in a MyD88-dependent fashion which augments T cell activation[102]. Due to their high capacity to induce the maturation of DCs, which in turn influences the development of T cell responses, synthetically derived TLR agonists have been extensively studied in order to adjuvant adaptive immune responses to exogenously delivered antigens, such as in immunotherapies or vaccination strategies.

1.2 Antigen-specific T cell responses

T cells are the effector arm of the adaptive immune system responsible for the elimination of infected or neoplastic cells as well as augmenting other immune responses. T cells develop from lymphoid progenitors derived from the bone marrow that migrate to the thymus where they enter distinct phenotypic and morphological stages of development to form a naïve T cell[103,104]. During their development, the TCRα and TCRβ gene segments in lymphoid progenitors or 'thymocytes' undergo somatic recombination to develop randomly generated antigen receptors with unique specificities[105]. These TCRs then recognise self-antigens via Ag-MHC complexes on thymic cortical cells and undergo a process known as thymic selection. The intensity and manner in which these thymocytes recognise antigen on these complexes determines their survival and maturation. T cells with TCRs that bind Ag-MHC complexes with a moderate avidity receive survival signals and are positively selected. In contrast, if the TCR binds with too strong of an avidity then the T cell will undergo programmed cell death (apoptosis), therefore limiting generation of auto-reactive T cells. Additionally, the ability of a TCR to interact with antigen loaded onto MHC-I or MHC-II is crucial in influencing their CD4 or CD8 single-positive commitment[106,107]. Following their maturation and selection, $CD4^+$ and $CD8^+$ T cells display distinct functional differences. $CD8^+$ T cells contribute mainly to sterilising immunity through recognition of foreign or aberrantly expressed antigens on MHC-I, resulting in targeted destruction of infected or neoplastic cells by the $CD8^+$ T cell[108–110].

In contrast to this, $CD4^+$ T cells undergo context-dependent differentiation into several effector program states upon recognition of Ag-loaded MHC-II, and in turn influence pathogen-specific innate and adaptive immune responses[111]. Importantly, similarly to

TLR signalling, $CD4^+$ T cells can also influence the state of APC maturation through the expression of co-stimulatory molecules which enhance the immunogenic presentation of antigen towards $CD8^+$ T cells[12,15,112,113]. Moreover, this $CD4^+$ T cell 'help' co-operates with TLR signalling synergistically to greatly improve the function of APCs[21,114]. The following sections will detail the importance of $CD8^+$ T cells in sterilising immunity as well as emphasise the role of $CD4^+$ T cells in augmenting these responses through their helper functions.

1.2.1 CD8+ T cells

$CD8^+$ T cells represent the cytotoxic arm of the adaptive immune system. Their main function is the recognition of viral or endogenously-derived antigens in the context of MHC-I. Alternatively, exogenous antigens may also be acquired during endocytosis or intracellular infection and provide MHC-I antigens through cross-presentation. Cognate interactions between the TCR and relevant antigen-loaded MHC-I, supplemented by co-stimulatory molecules on the APC such as CD86/80 and cytokines like IL-12, result in the promotion of T cell cycle progression, survival and acquisition of effector functions[115–118]. Once activated, these primed cytotoxic lymphocytes (CTLs) lose expression of lymph node homing molecules CD62L and CCR7, and gain enhanced expression of CD44 and S1P1, which supports extravasation of CTLs into infected peripheral tissues[119,120]. CTLs activated through their TCR exert targeted destruction towards infected cells *via* the release of perforin and granzyme B, which results in the induction of programmed cell death[121,122]. They also express effector cytokines like IFNγ and TNFα that further contribute to the local inflammatory immune response to given infections through priming of other immune responses[123].

Following their proliferative burst and elimination of infection, the remaining CTLs undergo a contraction phase where the majority of cells will undergo programmed cell death to resolve inflammation[124,125]. The remaining cells may then undergo differentiation into memory cells that gain increased expression of markers such as IL-7 receptor-α (CD127), which promotes long-term survival[124,126,127]. These $CD8^+$ memory T cells seed secondary lymphoid organs at low frequencies but are poised to recognise and expand upon re-encounter of relevant cognate antigen rapidly.

Owing to their highly specific and potent effector capacity, research into $CD8^+$ T cells has highlighted their critical role in the control or resolution of numerous viral pathologies. Studies in mice infected with lymphocytic choriomeningitis virus (LCMV) show a substantial expansion of cytotoxic viral antigen-specific $CD8^+$ T cells followed by generation of immunological memory[128,129]. Many other human viral infections show preferential expansion of $CD8^+$ T cells such as Epstein-Barr virus, cytomegalovirus and hepatitis B virus[130–132]. Furthermore, in humans, non-progressing patients infected with human immunodeficiency virus (HIV) show a preferential expansion of HIV-specific $CD8^+$ T cells that maintain perforin expression, highlighting the importance of CTLs in limiting viral replication[133]. $CD8^+$ T cells also represent a highly significant subset in anti-tumoural immunity. Numerous studies across a variety of cancer types including colorectal, ovarian and breast cancer in humans have shown the presence of $CD8^+$ tumour infiltrating lymphocytes (TILs) as a prognostic factor for increased survival in patients[134–136]. Moreover, a meta-analysis of over 50 research papers showed that $CD8^+$ TILs were a positive prognosis factor with a hazard ratio (HR) of 0.71[137].

The overwhelming body of research details the imperative role that $CD8^+$ T cells play in both viral and anti-tumoural immunity. Therefore, adjuvants that enhance APC function to allow the generation of potent antigen-specific $CD8^+$ T cells are critically important and are desirable targets for the development of immunotherapies.

1.2.2 CD4+ T cells

$CD4^+$ T cells are specialised mediators of immunity against bacterial, fungal and parasitic infections. Upon recognition of exogenous antigen on MHC-II, $CD4^+$ T cells may undergo further differentiation into T helper (Th) subsets identifiable by unique transcriptional and cytokine profiles. Importantly, this differentiation is determined by the local inflammatory environment with each subset providing tailored responses to the given pathogen. For example, during intracellular infection, activated APCs that produce IL-12 drive $CD4^+$ T cell polarization to a 'Th1' phenotype. Downstream signalling through signal transducer and activator of transcription 1 (STAT1) and STAT4 results in expression of the T-box transcription factor (Tbet)[113,138]. This master regulator of the Th1 response governs downstream expression of the inflammatory cytokine, IFNγ, which induces enhanced phagocytic functionality of phagocytes such as macrophages[139]. Therefore, Th1 cells represent an essential T cell subset in the elimination of intracellular

pathogens. In response to helminth infection, expression of the transcription factor GATA is induced in $CD4^+$ T cells which governs the production of IL-4, IL-5, and IL-13. These 'Th2' cells ultimately facilitate IgE production by B cells, eosinophil recruitment, and mucous production[140]. Responses involving Th2 cells are inappropriately initiated against otherwise innocuous antigens in allergy. Extracellular bacterial or fungal infections induce RORγt expression and differentiation into the Th17 phenotype, which through the production of IL-17, IL-6, IL-8, and TNF, mediate recruitment of neutrophils and monocytes[141,142].

1.2.2.1 CD4+ T cell help

Amongst their immune-modulating effects, $CD4^+$ T cells are also key in augmenting B cell responses. After activation, $CD4^+$ T cells migrate to the edge of the B-cell follicle within lymph nodes where they may encounter and activate cognate antigen-presenting B cells to induce germinal centre formation[13,17]. The first evidence detailing the requirement of T cells in B cell activation were early cell transfer experiments in irradiated mice. These studies revealed that it was only when cells from both the thymus and bone marrow were simultaneously transferred back into mice that antibody responses could be generated after immunisation[143,144]. It was later found that efficient B cell activation required stimulation through both the B cell receptor (BCR) and CD40 (a member of the tumour necrosis factor family; TNF) driving germinal centre formation and promoting B cell proliferation and survival[145]. The ligand for CD40, CD40L, is expressed highly on activated $CD4^+$ T cells[146]. Contact dependent co-stimulation of B cells through CD40L together with inducible T cell co-stimulator (ICOS) and SLAM-associated protein (SAP) all contribute to providing help to B cells, as gene deletion of these proteins leads to severe defects in germinal centre formation and B cell memory[17,147,148].

Furthermore, humans who have X-linked hyper IgM syndrome contain mutations in *CD40LG* and cannot form germinal centres[149]. Upon ligation of CD40 to CD40L, several of TNF receptor-associated factors (TRAFs) induce signal transduction and downstream expression of transcription factors, such as NFκB, triggering B cell activation, survival, cytokine production, and class-switching of antibody[150–154]. In particular, a subset of $CD4^+$ T cell, the T follicular helper (T_{fh}) cell are poised to provide help to B cells. These T_{fh} cells can migrate into the B cell zone due to increased expression of the chemokine

receptor chemokine (C-X-C motif) receptor 5 (CXCR5)[155]. Within early germinal centres, these cells provide signalling through their constitutive expression of CD40L and ICOS as well as expression of the cytokine IL-21[156].

Aside from B cells, CD40 is expressed on a number of other immune cells including monocytes, macrophages, and DCs where T cells are key providers of 'help' signal. In this way, $CD4^+$ T cells are crucial in the activation and maturation of DCs and subsequent priming of potent $CD8^+$ T cell responses. In a similar manner to CD40-CD40L signalling in B cell activation, this signal cascade in DCs leads to transcriptional changes resulting in the maturation of the DC. Recognition of cognate antigen between a $CD4^+$ T cell and a DC leads to upregulation of co-stimulatory markers CD86/80 as well as heightened expression of IL-12 leading to enhanced interactions between $CD8^+$ T cells and DCs[14–16]. Importantly, efficient $CD4^+$-dependent priming of $CD8^+$ T cells requires antigen to be recognised by both subtypes on the same APC[15,157,158]. Numerous early studies *in vivo* have highlighted the necessity of the presence of $CD4^+$ T cells in priming antigen-specific $CD8^+$ T cells[158–160]. Moreover, the formation and maintenance of immunological memory to infection requires the presence of $CD4^+$ T cells[112,161,162].

It is now also clear from genomic and transcriptomic studies that CD4+ T cell help instructs $CD8^+$ T cell fate through induction of differential transcriptional pathways. In an RNA-sequencing study, mice were vaccinated with one of two DNA vaccines. The first utilised the oncogenic human papillomavirus (HPV) E7 protein that encoded for the MHC-I peptide alone. The second utilised both the E7 protein and an unrelated HPV known MHC-II epitope, which would allow for expression and co-localisation of MHC-I and II epitopes within a single APC[12]. The authors found that the CTLs generated in the presence of helper antigens had higher expression of TNF, IFNγ, FASL, and Granzyme A/B, which conferred higher cytotoxic qualities in further studies[163]. Furthermore, these CTLs showed higher expression of CXCR4, CX3C-chemokine receptor 1 (CX3CR1) promoting tumour extravasation. In contrast, CTLs that arose from no T cell help had higher expression of inhibitory proteins programmed cell death protein 1 (PD-1) and lymphocyte activation gene 3 protein (LAG3). These $PD\text{-}1^+$ CTLs are reminiscent of those found in human cancers formed during early stages of effector differentiation and display an 'exhausted' dysfunctional phenotype which would suggest that exhausted CTLs may arise from lack of helper signals[164].

These studies emphasised the biological impact of $CD4^+$ T cell help, especially within the context of cancer. Importantly, this study highlighted the fact that $CD4^+$ T cell epitopes did not need to be a tumour-specific antigen to elicit help. For this reason, many studies utilising MHC-I epitopes in vaccination studies often include MHC-II epitopes (reviewed here[165]). Alternatively, agonistic proteins to induce helper signalling has been used to bypass the requirement of antigen-specific $CD4^+$ T cells. In a therapeutic cancer vaccine model, combination with agonistic CD40 antibody (αCD40) overcame peptide-induced tolerance and greatly enhanced anti-tumour activity[166]. Collectively, there is substantial evidence on the importance of CD40 signalling in the development of potent T cell responses, and accordingly, αCD40 has been employed as a combinatorial therapy in many cancer trials (reviewed here[167]).

1.2.3 NKT cell help

Within a host's diverse repertoire of T cells, with each cell population expressing multiple copies a single randomly generated TCR structure, there also exist subpopulations of T cells that display limited TCR diversity and therefore bind to a limited repertoire of antigens. These T cells generally have a "semi-activated" phenotype, and so respond very quickly (within hours) to exposure to antigen. They also commonly express markers typical of natural killer cells – fast-acting cytotoxic cells of the innate immune system. They have therefore been termed "innate-like" T cells. One such subset of innate-like T cells, NKT cells, have been extensively studied and have potent anti-tumour properties. NKT cells are selected in the thymus by an MHC class I-like molecule, CD1d[168,169]. This molecule presents an array of lipid antigens to NKT cells which are recognised by their invariant TCR (Vα14-Jα18 in mice and Vα24-Jα18 in humans). One of their most potent known agonists, α-GalCer, activates NKT cells and elicits rapid activation and production of an array of cytokines including large amounts of IFNγ and IL-4, key in driving Th responses. In fact, α-GalCer was originally discovered as an anti-tumour agent with potent single-agent effects. *In vitro* studies established that NKT cells themselves were capable of direct lysis of tumour cells when α-GalCer was loaded onto CD1d-expressing mouse or human tumour lines[170,171]. These findings were seemingly confirmed *in vivo*. However, further observations across models showed that the potency of α-GalCer was more related to activation and modulation of other arms of the immune system[172,173]. Indeed, α-GalCer-dependent tumour suppression was found to be dependent on IFNγ as

well as IL-12 producing DCs, an essential mechanism in the activation of natural killer (NK) cells and priming of CTLs[174–176]. Their pronounced ability to induce IL-12 secretion by DCs suggests that NKT cells could be acting as mediators of help, similar to $CD4^+$ T cells.

Further studies confirmed that mice injected with α-GalCer show strong activation of DC populations displaying increased levels of maturation markers CD86/80 and high levels of IL-12[19,21]. This effect was mainly mediated by contact-dependent stimulation through the CD40-CD40L axis. These immune-stimulating properties of activated NKT cells raised the possibility that α-GalCer could be used as an immune adjuvant to enhance priming of antigen-specific CTLs. This approach was attractive as NKT cells represent a highly frequent population of T cells, around 0.5% of circulating T cells in mice and can also be found highly enriched at secondary lymphoid tissues as well as distal tissues like the liver and lungs[177]. Indeed, co-administration of α-GalCer with model antigens showed that NKT cells could induce the proliferation of antigen-specific CTLs, which could confer significant protection against tumour challenge in a CD1d-dependent manner[18–20].

Moreover, the helper function of NKT cells has been highlighted across numerous disease settings. In murine models of malaria, administration of α-GalCer to mice was able to inhibit the development of the malarial parasites *Plasmodium yoelii* and *Plasmodium berghei*[178]. Intranasal administration of α-GalCer during murine influenza infection resulted in IL-4 dependent production of IgA[179]. Moreover, the role of NKT cells in anti-viral immunity was confirmed in a porcine model of influenza where administration of α-GalCer resulted in inhibition of viral replication[180]. Importantly, NKT cells have also been shown to induce the expansion of $CD8^+$ T cells specific for human tumour associated antigens *in vivo* when $A2K^b$ transgenic mice were co-administered α-GalCer and NY-ESO, highlighting the great therapeutic potential for clinical trials.[181]

The anti-tumour benefits of NKT cell-based immunotherapies within the human setting remains unclear. A number of clinical trials across various cancers have been conducted where α-GalCer is either loaded onto autologous immature monocytes or mature DCs and infused back into the patient (reviewed here[182]). In many instances, NKT cells within the patients in these trials were found to be highly activated post-infusion, producing IFNγ and in some cases were accompanied by broad inflammatory responses including the

expansion and activation of T cells and NK cells. However, thus far, these NKT cell-based therapies have shown little to no anti-tumour effects. Despite these numerous trials, little emphasis has been placed on the ability of NKT cells to adjuvant $CD8^+$ T cell responses through CD40L-dependent help of DCs. However, one study found that infusion of patients with α-GalCer pulsed DCs resulted in the expansion of cytomegalovirus (CMV)-specific $CD8^+$ T cells, providing evidence that NKT cell-based helper function could occur in the human setting[183]. A recently completed phase I clinical trial by our laboratory aimed to address this property of NKT cells further through the administration of autologous DCs loaded with α-GalCer and tumour-associated antigens; MELVAC study[184]. In this study, NKT cells in most patients were found to proliferate and produce pro-inflammatory cytokines post-vaccination. Moreover, seven out of eight patients showed an increase in polyfunctional antigen-specific T cells providing evidence that the NKT-helper function may indeed be conserved in humans. However, more research is required to fully underpin the capacity of NKT cells to act as cellular adjuvants to improve the quality of anti-tumour responses. One possible limitation in NKT cell-based immunotherapies, however, is the difference in frequency between mouse and humans. In contrast to mice, NKT cells in humans only represent up to 0.05% of circulating T cells[22] which raises concern in the translation of NKT cell-based therapies in the clinic.

1.3 MAIT cells and their potential as immune adjuvants

MAIT cells are another innate-like T cell group that are functionally reminiscent of NKT cells. They express an invariant TCR that recognises derivatives of the vitamin B2 biosynthetic pathway produced by bacteria or fungi on an MHC I-like molecule, MHC I-related gene 1 (MR1)[185]. Moreover, MAIT cells can react to viral infections and become activated in an MR1-independent manner through cytokines such as IFNα, IL-12 and IL-18[186]. In contrast to NKT cells, MAIT cells are an abundant population of T cells in humans, representing 1-10% of circulating T cells[23,25]. They are enriched within secondary lymphoid organs, the liver, and at mucosal sites such as the lamina propria and the lungs. Since their discovery, MAIT cells have been highlighted as sensors of bacterial infection. Studies in $MR1^{-/-}$, which lack MAIT cells, show defects in early protection against models of bacterial infection, however, do not seem to be essential for sterilizing immunity[26,187]. Amongst vertebrates, MAIT cells are highly conserved and therefore must

have essential and non-redundant functions aside from their anti-microbial properties that are yet to be described. Emerging evidence has indicated that MAIT cells also play a role in immune regulation and modulation[27,29,188,189]; however, the extent of this is not fully understood. This section will detail the current knowledge of MAIT cell biology, their known agonists, and explore their potential as immune adjuvants which may overcome the limitations of NKT cell research in humans.

1.3.1 MAIT cell development and phenotype

Like conventional T cells, MAIT cells develop in the thymus from bone-marrow derived lymphoid progenitors. Once in the thymus, cells expressing the invariant MAIT TCR, Vα19-Jα33 in mice and Vα7.2-Jα12/20/33 in humans, are selected for by MR1 (ligand to be discussed later)[190,191]. They express limited Vβ chain diversity being mainly Vβ2 and Vβ13.2 in humans and Vβ6 or Vβ8 in mice[23,192,193]. In humans, the dominant population of MAIT cells are $CD8^+$ but can be double negative (DN) for expression of CD8 and CD4, with a small population of $CD4^+$ cells[23,25]. In contrast, the majority of murine MAIT cells are DN with small populations being $CD8^+$ or $CD4^+$[23]. During late stages of development, MAIT cells acquire expression of the transcription factor promyelocytic leukaemia zinc finger protein (PLZF), akin to other innate-like T cells, including NKT cells, Vγ9Vδ2 T cells, and innate lymphoid cells[194–197]. This transcription factor defines the innate-like T cell group as the expression of PLZF drives the acquisition of an effector phenotype through induction of lineage defining transcription factors such as T-bet, RORγt, and GATA3[195]. In the case of MAIT cells, the most commonly expressed factor is RORγt, making them phenotypically comparable to Th17 cells, especially within the lung, lamina propria and the skin with >80% being $ROR\gamma t^+$ MAIT cells[23,198]. MAIT cells may also express T-bet, however, to a lesser extent than RORγt, only representing minor populations within the blood, spleen, lymph node, and liver. Interestingly, only a very small proportion of MAIT cells express GATA3, which are only detectable in B6-$MAIT^{CAST}$ mice. This strain on the C57BL/6 background harbours high MAIT numbers due to congenic expression of a genetic locus derived from CAST/EiJ mice that confers higher expression of the Vα19-Jα33 TCR arrangement[198]. For ease, $ROR\gamma t^+$ MAIT cells and $T\text{-}bet^+$ MAIT cells will be referred to as MAIT17 and MAIT1, respectively. Owing to the over-representation of MAIT17 cells, upon stimulation, the MAIT cell population produces high levels of IL-17 and low levels of IFNγ and IL-4, distinctive from NKT cells[23,25]. Analogous to CTLs, MAIT cells also produce perforin and granzyme B during

activation. Upon recognition of antigen-loaded MR1 on infected cells MAIT cells rapidly degranulate and release these effector molecules to induce apoptosis[199]. Although human and mouse MAIT cells highly overlap in their phenotype and functionality, one striking difference is that human MAIT cells can represent up to 10% of circulating T cells, as well as 45% of T cells within the liver[25,200]. In contrast, in mice, MAIT cells are far less represented at these locations. In mice, MAIT cells account for less than 0.1% of circulating T cells and reach up to 1% of T cells within the lungs. As MAIT cells react to bacteria-derived metabolites, and most laboratory mice are specific-pathogen free (SPF), this may be a consequence of evolutionary negative selection pressure against the need for high frequencies of MAIT cells.

1.3.2 MAIT ligands and MR1

The MHC I-related molecule, MR1, is notably one of the most conserved MHC-I proteins across mammals with 90% sequence homology between the α1 and α2 domains within the murine and human *Mr1* genes[201,202]. As a comparison, between murine and human CD1d, the selecting and presenting molecule for NKT cells, only shares about 60% sequence homology. In a similar manner to classical MHC-I molecules, MR1 is ubiquitously expressed amongst tissues and is synthesised and localised within the ER where it associates with β_2-microglobulin (β2m)[203]. In contrast to MHC-I molecules, MR1 does not bind to peptide antigens, evident by the lack of commonly conserved residues found in MHC-I molecules involved in peptide anchoring[203]. Instead, MR1 binds to small pyrimidine intermediates that are formed as a by-product of microbial riboflavin (vitamin B2) synthesis[185]. Specifically, the intermediate 5-A-RU, which was found to activate MAIT cells, does not directly bind to MR1 itself but instead undergoes enzymatic condensation with α-dicarbonyl species. Methyglyoxal, glyoxal and butane-2,3-dione react with 5-A-RU to form the ligands 5-OP-RU, 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU) and 5-(1-methyl-2-oxopropylideneamino)-6-D-ribitylaminouracil (5-MOP-RU), respectively[185,204]. For simplicity, studies on 5-OP-RU will be the main focus of discussion here. During intracellular infection or phagocytosis of bacteria, it is thought that 5-A-RU condenses with methylgloxal, which is produced as a product of cellular metabolism in order to form 5-OP-RU. This molecule then covalently bonds to ER-localised MR1 through Schiff base formation *via* a lysine residue (K43) located within the antigen-binding groove[185]. Binding of 5-OP-RU to MR1 induces conformational change and subsequent translocation to the cell surface for presentation

to MAIT cells[205]. Activation of MAIT cells is then induced through direct hydrogen bond interactions between the CDR3α loop on the MAIT cell TCR and the ribityl moiety on 5-OP-RU[206]. It is important to note that MR1 does show some level of versatility in the antigens that it binds. For instance, a screen of small molecules showed that MR1 could bind drug-like molecules and metabolites, which can activate MAIT cells[207,208].

The vitamin B9 metabolite, 6-formylpterin (6-FP), is another ligand that is able to form a Schiff base with MR1, induce conformational change and egress to the cell surface[206,209]. However, in contrast to 5-OP-RU, 6-FP lacks the necessary moieties to contact the MAIT TCR and therefore, acts as an antagonistic ligand by sequestering MR1 molecules and inhibiting binding with other activatory ligands. Thus, 6-FP and its synthetic derivative, acetyl-6-formylpterin (Ac-6-FP), are useful tools in studying the mode of MR1 presentation (discussed in 1.3.3.2).

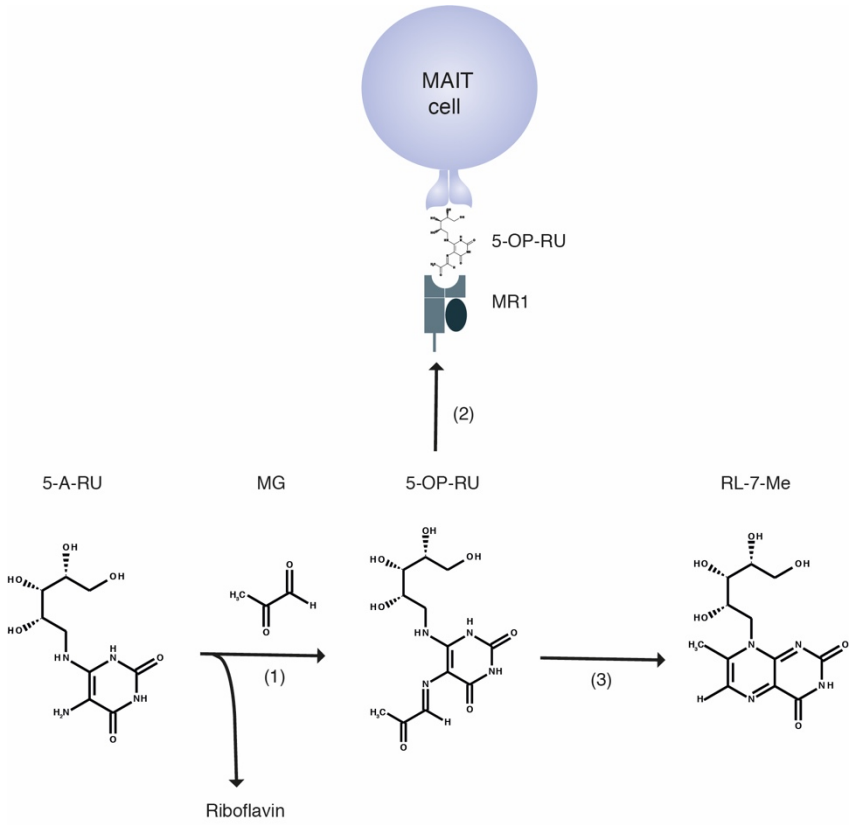


Figure 1.1. Formation of MR1 ligands to activate MAIT cells. (1) During microbial riboflavin synthesis, 5-A-RU undergoes condensation with dicarbonyl adducts such as MG to form 5-OP-RU. (2) This pyrimidine intermediate forms a Schiff base with K43 on MR1 and is then presented to the MAIT cell TCR, inducing activation. (3) However, 5-OP-RU is also susceptible to cyclisation into 7-methyl-8-$_D$-ribityllumazine which is a weak MR1 ligand.

1.3.2.1 MAIT agonist instability

One of the limitations of research utilising MAIT agonists is their propensity to rapidly cyclise into inactive lumazines (Figure 1.1)[185,204]. The formyl group located on 5-OP-RU is a crucial binding site for covalent bonding to MR1. Thus, cyclisation of this group in the formation of lumazines precludes this site from bonding to MR1, making it a poor activator of MAIT cells. Moreover, in aqueous solution at 15^{o}C, 5-OP-RU has a half-life of 14 h (pH 6.8), 5-OE-RU 18 min, and 5-MOP-RU 14 h, both at pH 8[210]. The formation of 5-OP-RU is normally achieved by adding MG to 5-A-RU prior to use. However, in aqueous solution, this has been reported to be highly inefficient, with only around 1% conversion to 5-OP-RU, and rapid degradation with a half-life of 12 min. These issues have been overcome when 5-A-RU was reacted with MG in DMSO and under argon, which displaces air and prevents oxidation, enhancing 5-OP-RU conversion and stability[210]. In the same study, the authors also attempted to chemically modify 5-OP-RU in order to impede lumazine formation. Unfortunately, this was not highly successful as

the chemical variants of 5-OP-RU were drastically reduced in their ability to activate MAIT cells, possibly due to altered MR1 presentation. The precursor 5-A-RU has also been reported to succumb rapidly to degradation upon exposure to light or air, although these degradation products and their ability to activate MAIT cells have not been explored[211], which is a topic of this thesis. In this degradation study, 5-A-RU could be stabilised through the preparation of 5-A-RU as an HCl salt which protected the compound from degradation at room temperature for a maximum of 37 d in solid form when protected from light. However, it was unclear from the results of this study, whether stabilisation of 5-A-RU was able to confer reproducible and enhanced activation of MAIT cells compared to native 5-A-RU.

While these studies successfully provided methods to stabilise MAIT cell antigens for storage and use *in vitro*, no data has yet been published that detail modifications which result in the enhancement of MAIT cell activation *in vitro* or *in vivo*. During bacterial infection, 5-A-RU is produced *in situ* by bacteria present within the cell and therefore in close proximity to MR1. As of yet, it is unclear if the integrity of MAIT antigens is compromised by proteins or metabolites present *in vitro* within cell cultures, or *in vivo* within serum or extracellular spaces prior to uptake by APCs. Therefore, the generation of a modified 5-A-RU that both increases stability and prevents the formation of putative degradation products prior to cellular uptake would be a welcome advance within the MAIT cell field in order to explore this possibility. This is another topic of this thesis.

1.3.2.2 The mode of MR1 presentation

The canonical mode of MR1 presentation occurs in a similar manner to most MHC-I molecules and requires association with β2m to form stable complexes[212]. In a TAP-independent manner, MR1 is loaded with 5-OP-RU within the ER, which results in trafficking to the cell surface. After contact with the MAIT cell TCR to prime activation, MR1 becomes internalised within endosomes that consequently enter the degradation pathway through fusion with lysosomes. However, a proportion of these MR1 molecules are also able to enter the recycling pathway[205,213]. In this way, endosomes containing MR1 can acquire antigens by fusion with incoming endocytic vesicles containing phagocytosed bacteria. Evidence for the requirement of such pathways were detailed in studies comparing MR1 presentation between mycobacterial supernatant, containing exogenous soluble MAIT cell antigens, and whole mycobacteria, which provide

intracellular/endogenous sources of MAIT cell antigens. In these studies, cells loaded with the MR1 ligand 6-FP, blocked MR1 presentation and MAIT cell activation by mycobacterial supernatant but not when whole mycobacteria were used[214,215]. Genetic silencing of pathways involved in ER-Golgi organisation and endosomal trafficking revealed different requirements for exogenously provided soluble antigen versus endogenous bacterial derived antigen. Additionally, access of recycled MR1 molecules by soluble MAIT antigens was confirmed in a 2016 study. Here the authors showed that once MR1 was allowed to accumulate on the cell surface by incubation of cells with 6FP, blocking egress of ER-derived MR1 with brefeldin did not completely inhibit loading of MR1 when the cells were loaded with 5-OP-RU[205]. However, the biological implications of the existence of ER vs recycled MR1 presentation have not been fully explored.

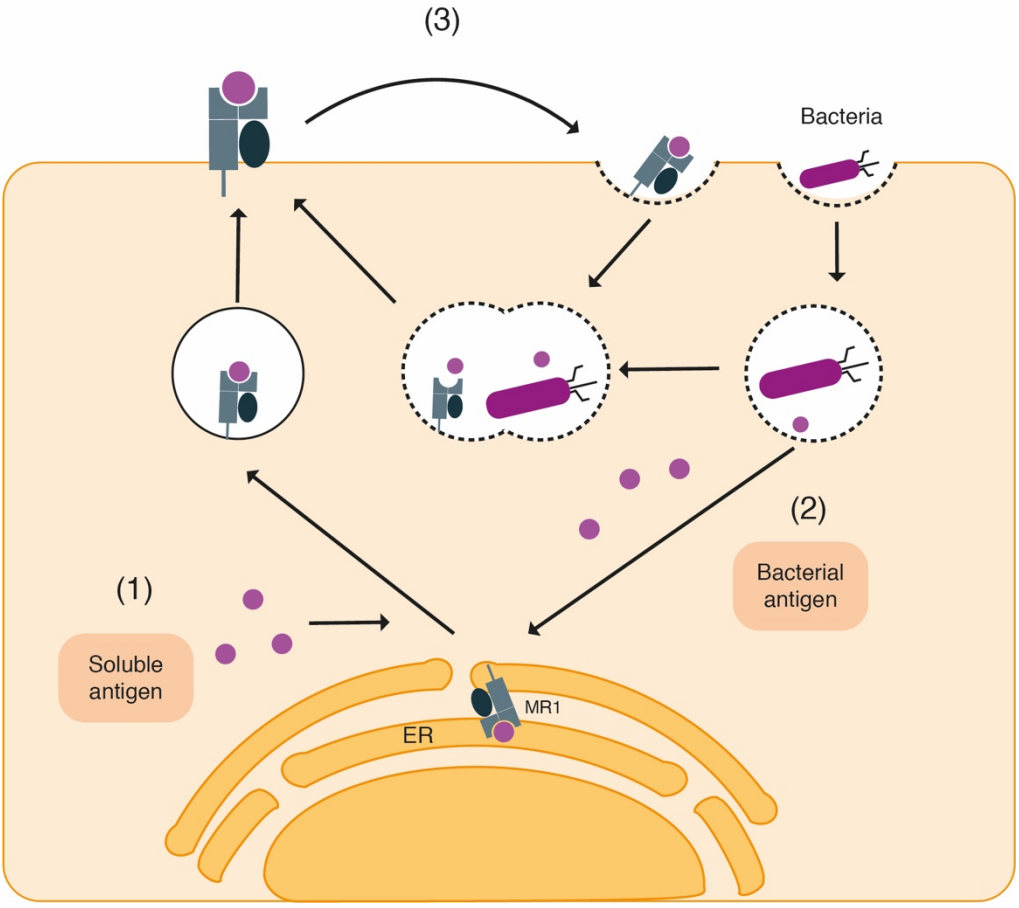


Figure 1.2 Mode of MR1 presentation. (1) Loading of an unknown endogenous or exogenously provided soluble antigens occurs in the ER where formation of a Schiff base with K43 in the MR1 binding groove induces translocation of MR1 through the trans-Golgi network to the cell surface for presentation. (2) Bacterially derived MAIT antigens can also provide ligands to ER-localised MR1. (3) However, MR1 can also enter recycling endosomes after presentation on the cell surface, which could potentially fuse with incoming endocytic vesicles containing bacteria, thus providing a separate loading mechanism for exogenous antigens.

1.3.3 Role of MAIT cells as anti-microbial effectors

Evidenced by the high evolutionary conservation of MR1 across mammalian species and its propensity to bind by-products of bacterial riboflavin synthesis produced by bacteria and some yeasts, MR1-restricted MAIT cells are a significant subset of T cells poised to react to bacterial infection. Early research has shown *in vitro* that MAIT cells can directly react to and lyse bacterially infected cells, as well as some yeasts species[199,216,217]. The ability to control bacterial infection is also reflected in *in vivo* studies. Mice deficient for MR1, and therefore lacking MAIT cells, succumb to disseminated infection by *Klebsiella pneumoniae* following intraperitoneal challenge compared to WT controls[187]. Similarly, sublethal intranasal infection with *Francisella tularenis*, resulted in preferential

expansion of MAIT cells in the lungs during early and intermediate stages of infection. MR1 deficient mice showed significant decreases in levels of pro-inflammatory cytokine within the lungs such as IFNγ, TNF, and IL-17 and failed to control bacterial growth resulting in delayed clearance of disease[26]. Additionally, this study showed MR1 deficient mice had reduced levels of activated $CD8^+$ and $CD4^+$ T cells infiltrating the lungs during infection compared to wild type, inferring that MAIT cells may also play a role in the modulation of other adaptive immune responses. Another study in MR1 deficient mice infected with Bacille de Calmette et Guérin (BCG) showed higher bacterial loads at early stages of disease compared to wild type[218]. Moreover, MAIT cells were shown to directly and potently inhibit intracellular growth of BCG-derived bacteria within macrophages. These studies show a role for MAIT cells in potentiating inflammatory immune responses *in vivo* for the control of bacterial disease.

In humans, reduced numbers of MAIT cells were commonly correlated with active diseases such as in patients with *Pseudomonas aeruginosa* infection (cystic fibrosis), tuberculosis and septic shock[216,217,219,220]. Moreover, patients whose MAIT cells remained at low frequencies after infection were more susceptible to subsequent hospital-acquired infections. Studies in mice showing an accumulation of MAIT cells within tissues during active infection suggest that this reduction in circulation is caused by direct recruitment of MAIT cells towards infected tissues from the periphery. However, more definitive research comparing MAIT cells from the tissue and peripheral blood is required in order to assess the role of MAIT cells in bacterial infections directly, and whether they are indeed homing to sites of infection.

1.3.4 MAIT cells as modulators of adaptive immune responses

The growing body of literature describes MAIT cells as potent effectors against bacterial infection, in which MAIT cells are able to accumulate within infected tissues where their primary function is assumed to be the control of bacterial growth through direct lysis of infected cells. However, as briefly described above, MAIT cells are producers of pro-inflammatory cytokines and are involved in the recruitment of activated T cells into the lung during pulmonary infection, suggesting that MAIT cells may be involved in modulating adaptive immune responses. The evidence for MAIT cells being involved in these responses are studies detailing their ability to interact with DCs. In one study, human MAIT cells were shown to drive the maturation of moDCs *in vitro*. In this study,

co-culture of MAIT cells and monocytes resulted in CD40L-dependent increased expression of the co-stimulatory markers CD80 and CD83 as well as the production of IFNγ and IL-12p40[29]. Moreover, MAIT cells were also able to activate primary DCs isolated from human blood, showing that they have the ability to interact with multiple subtypes of DC. Furthermore, in an *in vivo* study of mice infected with *F. tularensis,* MAIT cells could directly produce GM-CSF, an essential cytokine for DC development[26]. In this study, the production of GM-CSF by MAIT cells was key during infection to induce the recruitment and differentiation of monocytes into moDCs. Critically, MR1 deficient mice showed not only reduced recruitment of these moDCs but also a reduction in the number of $CD4^+$ T cells coming into the lung, which could be rescued by adoptive transfer of MAIT cells. It is likely that moDCs are acting as APCs in this model, priming the expansion of $CD4^+$ T cells. In another study, mice infected with *Legionella longbeachae* showed heightened expression of the co-stimulatory molecule, ICOS, on MAIT cells[221]. In this model, ICOS stimulation by MAIT cells induced the production of IL-23 (assumed to be from APCs) which drove the expansion of lung resident MAIT cells and subsequently conferred protection against infection.

The interactions between MAIT cells and B cells have not been explored in great detail. The first indication of MAIT-B cell interactions occurring was shown in mice that lack the immunoglobulin heavy-chain joining region ($J_H^{-/-}$). These mice were deficient for MAIT cells showing that B cells were important for MAIT cell development[222]. In a recent study, MAIT cells were shown to be able to influence B cell responses in a murine model of lupus[189]. In the mouse strain FcγRIIb$^{-/-}$*Yaa,* B cells spontaneously develop autoantibodies to nuclear antigens leading to inflammation across a variety of organs. In this study, when FcγRIIb$^{-/-}$*Yaa* were crossed with MR1$^{-/-}$, there was a marked reduction in autoantibody production and concurrent reduction in germinal centre responses. *In vitro* co-culture of MAIT cells with B cells and LPS lead to autoantibody production dependent on the presence of MAIT cells. Importantly, blockade of CD40L abrogated autoantibody production. This recent data reveals that MAIT cells are also able to provide CD40L signalling to B cells and may be a useful avenue to explore MAIT cells as cellular adjuvants towards generation of B cell responses, however, this possibility will not be explored in this thesis.

Overall the collected evidence presented here suggests that MAIT cells have the potential to modulate adaptive immune responses, which is likely to be relevant in disease pathogenesis and potentially provides an opportunity to develop novel immunotherapies. For this reason, the interaction of MAIT cells with APCs, and downstream effects on T cell responses, is a major theme of this thesis.

1.4 Rationale and aims

There is now growing evidence that MAIT cells are able to not only directly lyse bacterially infected cells but also influence the activation state of APCs. Moreover, the recent revelation that MAIT cells can provide co-stimulation to DCs through CD40-CD40L signalling indicates that, similar to NKT cells, activated MAIT cells may be harnessed therapeutically in vaccination strategies in combination with peptides.

At the initiation of this thesis, the ability of synthetic MAIT agonists to drive DC maturation, and potentially influence adaptive immunity, had not been explored in any great detail. During the course of the study, it was reported that MAIT cells activated by bacteria could induce DC activation in mice[27]. Also, when 5-A-RU is combined with MG to form 5-OP-RU, and then administered to mice intranasally, MAIT cells within the lung become activated[204]. Linking these observations together, it was hypothesised here that activation of MAIT cells in mice with synthetic cell agonists could be sufficient to lead to DC maturation. Furthermore, this process could potentially help drive T cell responses to co-administered antigens. If this hypothesis were correct, this information would provide supporting evidence that MAIT cell activities can shape T cell responses in disease, and could potentially be exploited in immunotherapy.

One of the limitations of research utilising these synthetic MAIT agonists is their poor stability and propensity to rapidly cyclise into inactive lumazines. Moreover, the degradation products of the precursor MAIT agonist, 5-A-RU, and their effects on MAIT cell activation have not been fully explored. Therefore, at the start of this thesis, these degradation processes were characterised, and an attempt was made to synthesise a stable version of 5-A-RU. Later in the thesis, the impact of activating MAIT cells *in vivo* with synthetic agonists was explored.

The specific aims of this thesis were to:

- Assess the degradation pathway of 5-A-RU when diluted in aqueous solution in preparation for biological use.
- Develop a stable “prodrug” form of 5-A-RU and assess the mode of release and presentation of this compound to MAIT cells

- Investigate whether activation of MAIT cells *in vivo* with synthetic ligands leads to enhanced activation status of DCs
- Examine whether co-administering or conjugating antigens with synthetic MAIT cell agonists will help prime antigen-specific T cell responses

Chapter 2:Materials and Methods

2.1 Buffers and solutions

Alsever's solution

Distilled H_2O with 20.5mgmL^{-1} dextrose, 4.2mgmL^{-1} sodium chloride and 8.0mgmL^{-1} sodium citrate (all BDH Laboratory Supplies, UK) adjusted to pH 6.1. Buffer was stored at 4^{o}C until use.

Fluorescent Activated Cell Sorting (FACS) buffer

Non-sterile PBS with 10mM ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich, MO, USA), 1% foetal calf serum (FCS; Gibco, ThermoFisher, MA, USA) and 0.01% sodium azide. Buffer was stored at 4^{o}C until use.

Iscove's Modified Dulbecco's Medium – human complete (huIMDM-5)

Gibco™ IMDM supplemented with 5% Human AB serum (Sigma-Aldrich, MO, USA), 2mM GlutaMAX™ (Gibco), 1% penicillin/streptomycin (Gibco) and 55µM 2-mercaptoethanol (ThermoFisher). Buffer was stored at 4^{o}C until use.

Iscove's Modified Dulbecco's Medium – murine complete (muIMDM-5)

Gibco™ IMDM supplemented with 5% FCS, penicillin/streptomycin and 55µM 2-mercaptoethanol. Buffer was stored at 4^{o}C until use.

Percoll gradient solution

Non-sterile PBS with 33% Percoll (GE Healthcare Bio-Sciences AB, Sweden) and 2.2% Alseiver's solution. Buffer was stored at 4^{o}C until use.

Roswell Park Memorial Institute 1640 – complete (RPMI-5)

Gibco™ RPMI 1640 supplemented with 5% human serum, 1% penicillin/streptomycin, 2mM GlutaMAX™, 2mM non-essential amino acids (NEAA; Gibco), 55µM 2-mercaptoethanol and 1000U/mL IL-2 (derived from in-house hybridoma). Buffer was stored at 4^{o}C until use.

Würzburger buffer

Sterile PBS with 1% FCS, 5mM EDTA and 0.1% DNase I (Sigma-Aldrich). Buffer was stored at 4^{o}C until use.

2.2 Mice

2.2.1 Maintenance and Ethics approval

All mice were bred and housed at the Malaghan Institute of Medical Research and used from 6 weeks of age. Animal protocols were approved by the Victoria University Animal Ethics Committee and performed according to institutional guidelines.

2.2.2 Mouse strains

C57Bl/6J mice were derived from breeding pairs purchased from Jackson Laboratories (Bar Habor, ME, USA). Stock no. 000664.

MR1$^{-/-}$ mice lack the α1 and α2 domain for the *Mr1* gene and thus do not produce functional MR1 proteins. As a consequence, MR1$^{-/-}$ lack MR1-restricted T cells including (Vα19-Jα133) MAIT cells. Breeding pairs for this strain were kindly provided by Vincenzo Cerundolo (WIMM, Oxford, UK).

CD1$^{-/-}$ mice have their *Cd1d1* and *CD1d2* loci replaced by a neomycin cassette and thus do not produce functional CD1d proteins. These mice lack CD1d-restricted T cells including (Vα14-Jα18) iNKT cells. Breeding pairs for this strain were purchased from Jackson Laboratories. Stock no. 008881.

Jα18$^{-/-}$ mice have the *Traj18* exons replaced by a neomycin cassette and thus lack functional Jα18 proteins. These mice cannot form a functional variable region within the iNKT cell T cell receptor and therefore lack iNKT cells. Breeding pairs for this strain were purchased from Jackson Laboratories. Stock no. 0030524.

CD11c-DTR^{eGFP} mice express a CD11c transgene containing an insertion of the human diphtheria toxin receptor (DTR) sequence and enhanced green fluorescent protein (eGFP) under the control of the CD11c promoter (*Itgax*). Administration of DT i.p to these mice results in selective depletion of CD11c expressing cells[223]. Breeding pairs for this strain were purchased from Jackson Laboratories. Stock no. 004509.

Clec9A-DTR mice express a contain an insertion of the human DTR sequence replacing the exon 1 of Clec9A. Administration of DT i.p to these mice results in selective depletion

of Clec9A expressing cells. Breeding pairs for this strain on a BALB/cByJ background were originally sourced from Dr Cristiane Ruedl (Nanyang Technological University, Singapore)[224]. Mice were then bred as heterozygotes with C57Bl/6J mice for experiments.

IL-12p40^{eYFP} mice have an internal ribosomal entry site containing an enhanced yellow fluorescent protein (eYFP) downstream of the IL-12p40 (*Il12b*) endogenous stop codon. This allows for visualization of IL-12p40 in myeloid cell populations during activation. Breeding pairs for this strain were purchased from Jackson Laboratories. Stock no. 006412.

2.2.3 Generation of CD11c-DTR^{eGFP} bone marrow chimeras

Mice older than 8 weeks of age were fasted for 24 h prior to exposure to radiation. Using a Gammacell® 3000 Elan irradiator (Best® Theratronics, Canada), mice were irradiated by exposure of 500cGy radiation twice, 3 h apart then transferred into ventilated cages. After 24 h, mice were given an intravenous (i.v.) injection of $1x10^7$ bone marrow cells from donor CD11c-DTR mice. Mice were maintained on drinking water containing $2mgmL^{-1}$ of neomycin trisulphate (Sigma-Aldrich) for three weeks and rested for a further five weeks prior to use in experiments.

2.2.4 *In vivo* depletion

DT derived from *Corynebacterium diptheriae* (Sigma-Aldrich) was reconstituted at $1mgmL^{-1}$ in distilled H_2O. Administration to mice was given at a dose of 15ng per gram of body weight and administered i.p. as indicated by each experiment.

2.3 Tissue extraction and processing

2.3.1 Blood

2.3.1.1 Peripheral white blood cells

Blood was extracted from mice older than six weeks of age via submandibular bleeding with a 4mm lancet. Approximately 100µL of blood was collected into a 1.5mL Eppendorf tube containing 200µL of PBS (+10mM EDTA). One mL of red blood cell (RBC) lysis buffer solution (Qiagen, Hilden, Germany) was added to each tube before incubating at 37^oC for 30 min. Tubes were centrifuged at 1500 x g for 4 min to pellet white blood cells.

Supernatant was discarded and the cell pellet was resuspended in 200µL of FACs buffer prior to staining for flow cytometry.

2.3.1.2 Serum

Blood was extracted from mice older than six weeks of age via submandibular bleeding with a 4mm lancet. Approximately 150µL of blood was collected into a 1.5mL Eppendorf tube. Blood was allowed to coagulate at room temperature for 3 h before centrifugation at 21,000 x g in order to separate red blood cells from serum. Serum was collected into a new 1.5mL tube and stored at -20^{o}C until application.

2.3.2 Bone Marrow

Mice were culled by CO_2 asphyxiation, followed by cervical dislocation and legs were removed. Skin, tissue and muscle were removed from each leg and the tibia and fibula were collected into ice cold IMDM. Bones were cleaned first in 70% ethanol then IMDM to remove residual tissue. To extract bone marrow, the ends of the bones were cut off and flushed through with a 24-gauge needle containing IMDM. Bone marrow was mechanically processed through a 70µM filter (Becton Dickinson, NJ, USA) and washed through with IMDM into a 50mL Falcon tube (ThermoFisher). Cells were pelleted by centrifugation at 250 x g for 10 min. Cells were resuspended in unsupplemented IMDM at $5x10^7$ cells/mL prior to i.v. administration to irradiated mice.

2.3.3 Liver

Mice were culled by CO_2 asphyxiation, followed by cervical dislocation. Using an 18-gauge needle, 10mL of ice-cold PBS was injected through the left ventricle of the mouse in order to perfuse the liver, removing circulating red and white blood cells. Livers were harvested into ice-cold PBS then mechanically processed and washed through a 70µM filter into a 50mL Falcon tube. Cells were pelleted by centrifugation at 572 x g for 4 min. Supernatants were removed and resuspended in 50mL of 33% Percoll gradient solution. Cells were pelleted by centrifugation at 322 x g for 10 min with the brake off. Supernatant, including the top layer of hepatocytes, was removed and cells were resuspended in 2mL of RBC lysis solution for 5 min at 37^{o}C. Cells were pelleted by centrifugation at 572 x g, supernatant discarded and resuspended in 200µL of FACS buffer prior to staining for flow cytometry.

2.3.4 Lung

Mice were culled by CO_2 asphyxiation, followed by cervical dislocation. Using an 18-gauge needle, 10mL of ice-cold PBS was injected through the left ventricle of the mouse in order to perfuse the lung, removing circulating red and white blood cells. Lungs were harvested into 1mL of IMDM containing 5mgmL^{-1} of Collagenase I (Gibco) and 0.25mgmL^{-1} of DNase I (Sigma-Aldrich) before cutting the tissue into small pieces and incubating at 37^{o}C for 30 min. Lung tissue was mechanically processed and washed through a 70µM filter into a 50mL Falcon tube. Cells were pelleted by centrifugation in at 572 x g for 4 min. Supernatants was discarded then cells were resuspended in 2mL of RBC lysis solution and incubated for 5 min at 37^{O}C. Cells were pelleted by centrifugation at 572 x g, supernatant discarded and resuspended in 1000µL of FACS buffer prior to staining for flow cytometry.

2.3.5 Lymph nodes

Mice were culled by CO_2 asphyxiation, followed by cervical dislocation. The mediastinal lymph node (mLN) was isolated and placed 500µL of IMDM containing 100µgmL^{-1} of Liberase™ TL (Sigma-Aldrich) and 100µgmL^{-1} of DNase I. Lymph nodes were broken up using a 25-gauge needle and then incubated for 30 min at 37^{o}C. After 25 min of incubation, EDTA was added to the digestion media at a final concentration of 10mM. Cells were collected and filtered through a 70µM filter into a 1.5mL Eppendorf tube. Cells were pelleted by centrifugation at 1500 x g for 4 min. Supernatant was discarded and the cell pellet was resuspended in 200µL of FACs buffer prior to staining for flow cytometry.

2.3.6 Spleen

Mice were culled by CO_2 asphyxiation, followed by cervical dislocation. Spleens were harvested into 1mL of ice-cold IMDM prior to processing. Spleens were mechanically processed and washed through a 70µM filter into a 50mL Falcon tube. Cells were pelleted by centrifugation at 572 x g for 4 min. Supernatants were discarded then cells were resuspended in 2mL of RBC lysis solution and incubated for 5 min at 37^{o}C. Cells were pelleted by centrifugation at 572 x g, supernatant discarded and resuspended in 1000µL of FACS buffer prior to staining for flow cytometry.

2.3.7 Cell viability

Cells were diluted in Trypan Blue dye (Gibco) and placed onto a haemocytometer. Viable cells were counted as those cells which had excluded the dye (white). Total cell number was determined as:

Total live cells = (Average cell count per area) x (dilution factor) x (10^4) x (total volume of cells)

2.4 Vaccine compounds

2.4.1 Generation of 5-A-RU

5-A-RU was synthesised and kindly provided as an aqueous solution from Prof. Gavin Painter (Ferrier Research Institute, Wellington, New Zealand) and stored at -80°C. 5-A-RU was diluted in PBS and kept on ice for immediate use to allow minimal auto-oxidation of 5-A-RU. For *in vivo* use, 5-A-RU was used at 180nmol per mouse unless stated otherwise. Where stated, methylglyoxal (MG) was added to diluted 5-A-RU at 10x the molar concentration and mixed for approximately 1 min before immediate administration to the mouse.

2.4.2 Generation of 5-A-RU prodrugs and conjugates

The 5-A-RU prodrugs and conjugates were synthesised and kindly provided by Prof. Gavin Painter (Ferrier Research Institute, Wellington, New Zealand) as a lyophilised powder. To solubilise the compounds, DMSO was added drop-wise up to 10mgmL^{-1} then diluted further to 1mgmL^{-1} in PBS. Stock solutions were aliquoted and stored at -20°C. For *in vivo* use 5-A-RU prodrugs or conjugates were used at 5nmol per mouse unless otherwise stated.

2.4.3 Filtration of 5-A-RU

Where stated, 5-A-RU was filtered using Ultrafree®-MC filter tube (Merck KGaA, Darmstadt, Germany) according to manufacturers' instructions. Briefly, up to 500µl of stock 5-A-RU was pipetted into the tube into the filtered chamber and capped. The tube was added to a centrifuge and spun for 1 minute at 12,000 x g. Filtered 5-A-RU was collected from the tube for experimental use or frozen at -80°C for later use.

2.5 *In vivo* treatments

2.5.1 Intravenous vaccination

Each compound was prepared at the indicated concentrations per experiment in a total of 200µL of PBS. Mice were warmed under a heat lamp, placed in a restrainer then administered 200µL of the indicated compound into the lateral tail vein.

2.5.2 Intraperitoneal antibody blockade

CD40L blockade

Mice were administered 500µg of anti-mouse CD40L (CD154) (clone MR-1; BioXCell, NH, USA) via intraperitoneal injection (i.p.) on d -1. On d0, mice were administered anti-CD40L i.p again and then administered 5-A-RU or 5-A-RU + MG via i.v. Control mice received the same dose of Armenian Hamster IgG (BioXCell).

TNFα blockade

Mice were administered 500µg of Etanercept (Enbrel®; Amgen, CA, USA) via intraperitoneal injection (i.p.) on d -1. On d0, mice were administered Etanercept i.p again and then administered 5-A-RU or 5-A-RU + MG via i.v. Control mice received the same dose of Human IgG (Privigen; CSL Behring, PA, USA).

IFNγ blockade

Mice were administered 500µg of anti-mouse IFNγ (clone XMG1.2; BioXCell, NH, USA) via intraperitoneal injection (i.p.) on d -1. On d0, mice were administered anti-IFNγ i.p again and then administered 5-A-RU or 5-A-RU + MG via i.v. Control mice received the same dose of Rat IgG (BioXCell).

IFNAR blockade

Mice were administered 200µg of anti-mouse IFNAR (clone MAR1-5A3; BioXCell, NH, USA) via intraperitoneal injection (i.p.) on d -1. On d0, mice were administered anti-IFNAR i.p again prior to i.v. with 5-A-RU or 5-A-RU + MG. Control mice received the same dose of Mouse IgG (BioXCell).

2.6 Adoptive transfer antigen specific T cells

2.6.1 Dynabead enrichment

Spleens were harvested from OT-I and OT-II mice and processed to a single cell suspension as described in section 2.3.6. Cells were washed with and resuspended in Würzburger buffer at $1x10^8$ cells/mL. Isolation of OT-I and OT-II cells was done with Dynabeads™ FlowComp™ mouse CD8 or CD4 kit, respectively (ThermoFisher), according to manufacturer's instructions. To OT-I and OT-II cells respectively, 50µL of FlowComp CD8 or CD4 antibody was added per $1x10^8$ cells and incubated at 4°C for 10 min. Cells were washed by adding 2mL of Würzburger buffer, pelleting cells by centrifugation at 572 x g for 4 min then resuspended in 1mL of Würzburger buffer. FlowComp Dynabeads was added to either cell population at 150µL per $1x10^8$ cells then incubated at 4°C for 15 min, mixing cells well every 5 min to ensure maximum binding of Dynabeads. Cells were then placed in a magnetic column for 1 min and the negative fraction was removed. Cells were removed from the magnet and washed in 1mL of Würzburger buffer then placed in the magnet for another min and the negative fraction removed. The bead-bound cells were resuspended in 1mL of FlowComp release buffer and incubated at room temperature for 10 min. Cells were placed in the column for 1 min to separate the now unbound Dynabeads from the positive cell fraction. The positive fraction was transferred to a new tube and washed in 10mL of PBS prior to adoptive transfer.

2.6.2 Adoptive T cell transfer

OT-I and OT-II cells were prepared as stated in 2.6.1. Viable cell count was performed as in section 2.3.7. OT-I and OT-II cells were resuspended in PBS at $1x10^5$ cells/mL and $5x10^6$ cells respectively. Equal volumes of OT-I and OT-II cells were combined and 200µl of the final mixture was injected intravenously into each mouse. The final concentration each mouse received was $1x10^4$ OT-I cells and $5x10^5$ OT-II cells.

2.7 Tumour cell culture and challenge

2.7.1 B16-OVA cell culture

B16-OVA tumour cells (kindly provided by Dr Dick Dutton, Trudeau Institute, NY, USA) (were cultured in Nunc™ EasYFlask™ Cell Culture Flasks (ThermoFisher) in muIMDM-5. Cell cultures were also supplemented with 1% 500µg/mL Geneticin (Gibco) to select for OVA expressing B16-OVA cells until ~70% confluence was reached. Adherent cells were harvested by incubation with TrypLE (Gibco) for 2 min at 37ºC. Cells were then washed twice with IMDM to remove FCS before tumour challenge.

2.7.2 TC-1 cell culture

TC-1 tumour cells (kindly provided by Dr. Wu from John Hopkins University, MD, USA) were cultured in Nunc™ EasYFlask™ Cell Culture Flasks (ThermoFisher) in RPMI-5. Cell cultures were grown until ~70% confluence was reached. Adherent cells were harvested by incubation with TrypLE (Gibco) for 2 min at 37ºC. Cells were then washed twice with RPMI to remove FCS before tumour challenge.

2.7.3 Tumour challenge

Tumour cells were harvested as described in 2.7.1 or 2.7.2 and viable cell count performed. B16-OVA cells were resuspended in IMDM at a final concentration of $5x10^6$ cells/mL and TC-1 cells were resuspended in RPMI at a final concentration of $1x10^6$ cells/mL. Cells were filtered through a 70uM filter to remove cell aggregates. Each mouse was administered 100µL of the final suspension subcutaneously into the left flank. Tumours were measured and monitored over time until a maximal limit of $200mm^2$ was reached. Mice were culled by cervical dislocation or CO_2 asphyxiation if mice showed any illness of reached the maximum tumour size.

2.8 Flow cytometry

2.8.1 Cell surface staining

Cells were obtained from the protocols outlined in previous sections and aliquoted into a 96-well plate. Cells were pelleted by centrifugation at 1000 x g for 2 min and the supernatant discarded. Prior to staining for flow cytometry, all human cells were pre-treated with 50µL of FACS buffer containing 1% Human IgG (Privigen, PA, USA) for 5 min at 4ºC to block non-specific FcR-mediated antibody staining. For mouse cells, 50µL

of FACS buffer containing 1% anti-CD16/32 Ab (24G2; prepared from hybridoma supernatant) was used. Cells were washed by pelleting the cells by centrifugation, resuspending in 200µL of FACS buffer, pelleting again and discarding the supernatant, all consequent wash steps were done this way. In some experiments, 1µL of the H-2Kb/SIINFEKL Pro5™ MHC Pentamer (ProImmune, , Oxford, UK) was added directly to the pellet, the plate vortexed vigorously and incubated for 10 min in the dark at room temperature. Without washing the cells, 50µL of PBS containing 1:1000 Zombie NIR™ was added to the cell pellet and incubated for 20 min in the dark at room temperature. Cells were washed once prior to addition of the cell surface antibody mix. Antibody mixes containing the appropriate cell surface markers were added to FACS buffer according to their pre-determined concentration then added to the cell pellet and incubated at 4^{o}C for 20 min. Cells were washed a twice then resuspended in 200µL of FACS buffer for flow cytometry. Flow cytometry was performed on the either the BD LSRII SORP (Becton Dickinson) or the Cytek Aurora (Cytek Biosciences; CA, USA) and data was analysed using FlowJo software (TreeStar; OR, USA).

Table 1. List of mouse fluorescently labelled antibodies

Target	Fluorophore	Clone	Supplier	Concentration
CD3ε	BV510	17A2	Biolegend	1:100
CD4	APC-H7	GK1.5	BD Pharmigen	1:600
CD8α	BV421	52-6.7	Biolegend	1:1000
CD11b	BV650	M1/70	Biolegend	1:900
CD11c	PE-Cy7	HL3	BD Pharmigen	1:200
CD25	PE/Dazzle™ 594	3C7	Biolegend	1:400
CD40	FITC	3/23	Biolegend	1:200
CD44	BV711	IM7	Biolegend	1:1000
CD45.1	FITC	A20	Biolegend	1:1000
CD45.2	APC-Fire 750	104	Biolegend	1:600
CD64	Alexa Fluor 647	X54-5/7.1	Biolegend	1:200
CD69	Super Bright 600	H1.2F3	eBioscience	1:300
CD80	BV421	16-10A1	Biolegend	1:400
CD86	APC R700	GL1	BD Biosciences	1:800
CD170 (Siglec F)	Alexa Fluor™ 700	1RNM44N	eBioscience	1:200

CD274 (PD-L1)	PE-Dazzle™ 594	10F.9G2	Biolegend	1:600
CD279 (PD1)	PE-Cy7	RMP1.30	Biolegend	1:600
CD317 (BST2)	PE	927	Biolegend	1:1000
CD326 (EpCAM)	BV605	G8.8	Biolegend	1:1000
Ly6C	BV570	HK1.4	Biolegend	1:300
Ly6G	BV711	1A8	BD Biosciences	1:600
MHCII	eFluor 450	M5/114.15.2	Invitrogen	1:300
NK1.1	BV650	PK136	Biolegend	1:600
Siglec H	PerCP-eFluor710	eBio440c	eBioscience	1:200
TCRβ	APC	H57-597	Biolegend	1:200
Vα2	PE	B20.1	eBioscience	1:1000
XCR1	PerCP-Cy5.5	ZET	Biolegend	1:200
Zombie NIR™			Biolegend	1:1000
CD1d-α-GalCer	PE	-	NIH tetramer core	1µl per pellet
MR1-5-OP-RU	BV421	-	NIH tetramer core	1:200
H-2Kb-SIINFEKL	PE	-	ProImmune	0.5µl per pellet

Table 2 List of human fluorescently labelled antibodies

Target	**Fluorophore**	**Clone**	**Supplier**	**Concentration**
CD3	BV510	UCHT1	Biolegend	1:100
CD19	APC-Cy7	HIB-19	Biolegend	1:100
CD137	BV421	4B4-1	Biolegend	1:1000
CD161	APC	HP-3G10	Biolegend	1:100
Vα7.2	PE-Dazzle™ 594	3C10	Biolegend	1:100

2.8.2 Fluorescence-activated cell sorting

Fluorescence-activated cell sorting (FACS) sorting was carried out on a Sony SH800S Cell Sorter to purify populations of MAIT cells from human PBMCs. Human PBMCs were purified according to the protocol in 2.9.1. To stain for MAIT cells, the antibodies Vα7.2 and CD161 were added directly to the cell pellet in a 50mL Falcon tube and incubated on ice for 20 min, mixing every 5 min to ensure even staining. Cells were washed then resuspended in FACS buffer at a final concentration of approximately $1x10^8$

cells/mL Once sorted, cells were washed in RPMI-5 before cell culture as detailed in 2.9.2.

2.8.3 VITAL assay

In order to study the ability of a given vaccine to induce cytotoxic antigen-specific immune responses, the VITAL assay was used as previously described[225]. Briefly, spleens from C57Bl/6J mice were harvested and processed into a single cell suspension as described in 2.3.6. Cells were divided equally into four tubes. All cells were pelleted by centrifugation at 572 x g and supernatant was discarded.

CellTrace™ CFSE labelled & SIINFEKL loaded cells

Three of the tubes were resuspended in 2mL of muIMDM-5 containing SIINFEKL ($OVA_{257-264}$) at one of the final concentrations: 50nM, 5nM or 0.5nM. All cells were allowed to incubate for 2 h at 37°C, vortexing the tubes every 30 min to ensure homogenous uptake of the SIINFEKL. Tubes were pelleted by centrifugation and washed in excess IMDM two times and once in PBS. The 50nM, 5nM and 0.5nM SIINFEKL loaded cells were resuspended in PBS containing 2.5µM, 0.5µM and 0.1µM of CellTrace™ carboxyfluorescein succinimidyl ester (CFSE; ThermoFisher), respectively. Cells were incubated at room temperature in the dark for exactly 7 min at which point 10mL of FBS was added to each tube to quench any extracellular dye. Cells were washed twice in muIMDM-5 then once in IMDM before a cell viability count was performed. Each of the cell populations were resuspended in a final concentration of $3x10^7$ cells/mL and kept on ice until further application.

CellTracker™ Orange peptide-negative cells

One tube of splenocytes was resuspended in pre-warmed muIMDM-5 containing a final concentration of 5µM chloro-methyl-benzoyl-aminotetramethyl-rhodamine (CellTracker™ Orange; ThermoFisher) and incubated at 37°C for 15 min. Cells were pelleted by centrifugation and resuspended in pre-warmed muIMDM-5 then incubated a further 20 min at 37°C. Cells were pelleted by centrifugation and washed twice in muIMDM-5 and once in IMDM. The labelled splenocytes were resuspended in a final concentration of $3x10^7$ cells/mL and combined with each of the CFSE-labelled splenocytes at equal volumes. Cells were mixed well and 200µL of the final cell mix was injected i.v. into C57Bl/6J mice ($6x10^6$ cells of each population per mouse).

Peripheral white blood cells were collected from the mice via submandibular bleeding as described in 2.3.1.1, 18 h post i.v. injection. Populations of CTO and CFSE labelled cells were analysed by flow cytometry. To calculate the cytotoxicity of a given vaccine, a ratio was calculated between the total number of CFSE labelled (antigen-positive) cells and the total number of CTO labelled (antigen-negative cells). The CFSE/CTO ratio in treated mice was then normalized to that of naïve mice. Cytotoxicity was expressed as a percentage of specific lysis using the following equation:

% = 100 – ((“Treated mice ratio” / “Naïve mice ratio”) x100)

2.9 Human *in vitro* cell culture

2.9.1 Human ethics

Human peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteers who have given written consent. This study was approved by the Otago University Ethics Committee reference number H15/114 and all experiments were performed with relevant guidelines and regulations. The research advisory group for Māori (RAG-M) was also consulted in consideration for potential Māori participants to ensure cultural requirements. The consent form, patient information sheet and RAG-M approval can be found in Appendix E.

2.9.2 Isolation of peripheral blood mononuclear cells

Human PBMCs were obtained through density centrifugation (Lymphoprep; Axis-Shield, Oslo, Norway) of heparinised venous blood as follows. In a 50mL Falcon tube 35mL of blood was carefully layered onto 12mL of Lymphoprep. Cells were centrifuged at 800 x g for 20 min at room temperature with no brake. The mononuclear interphase located between the top plasma layer and above the Lymphoprep layer was collected with a Pasteur pipette into a new tube containing RPMI. Cells were pelleted by centrifugation then washed twice with RPMI prior to use. For applications beyond the day of collection PBMCS were pelleted and resuspended in a mix of 10% DMSO (Sigma-Aldrich, MO, USA) and 90% FCS (Gibco) at a final concentration of $2x10^7$ cells/mL, and cryopreserved in liquid nitrogen until required. To assess activation of MAIT cells, PBMCs were thawed

by diluting in pre-warmed RPMI, cells were then pelleted by centrifugation and resuspended in huIMDM-5 medium.

2.9.3 Activation of human MAIT cells

Human PBMCs were isolated as in 2.9.2 then cultured at $5x10^5$ cells/well in a Nunc™ MicroWell™ 96-well microplate (ThermoFisher). The MAIT agonists were added at concentrations specified in the text. Where stated, some cultures were pre-incubated with 1μM of Ac-6-FP (Cayman Chemical, MI, USA) for 2 h before adding the agonists. After 18 h, cell cultures were analysed by flow cytometry to assess MAIT cell activation.

2.9.4 Generation and culture of THP-1 mutant cell lines

The THP-1 mutant cell lines: THP-MR1, THP-GPI and THP-K43A were generated and kindly provided by Vincenzo Cerundolo (Oxford, UK). Briefly, to generate THP-1 mutant cell lines the endogenous MR1 locus was first deleted in THP-1 cell lines by CRISPR-Cas as previously described[226]. These MR1 depleted THP-1 cells were transduced with lentiviral particles generated in 293T cells transfected with lentiviral vectors encoding for full length human MR1 (GenBank AJ249778.1), MR1 K43A[227] or GPI-linked MR1[228]. THP-1 cell lines and variants were maintained in 6-well Nunc™ Cell-Culture plates (ThermoFisher) in RPMI-10.

2.9.5 MAIT cell agonist presentation

Human MAIT cells were isolated by FACS according to the protocol in 2.8.2 on PBMCs isolated from buffy coats purchased from Oxford Blood Donor Centre (Oxford, UK). Cultures of purified human MAIT cells were maintained in 6-well plates for up to 30 days in RPMI-5 at 37ºC with 5% CO_2. To assess presentation, $2x10^4$ MAIT cells were co-cultured with $5x10^4$ THP-1, THP-MR1, THP-GPI or THP-K43A in a 96-well plate at a final volume of 100μL combined. Where stated, increasing doses of Bafilomycin A1 (Sigma-Aldrich) was added to cultures for 2 h prior to the addition of 100μL containing the indicated MAIT cell agonists. After 24 h, supernatants were harvested and assessed for IFN-γ production via enzyme-linked immunosorbent assay detailed in 2.9.5.

2.9.6 Enzyme-linked immunosorbent assay (ELISA)

To assess IFNγ production from cell supernatants, enzyme-linked immunosorbent assay (ELISA) was performed as follows. To a 96-well ELISA Microplate (Greiner Bio-one, Austria) 25µL of ELISA coating buffer containing 0.4% anti- IFNγ capture antibody (BD biosciences) was added and incubated overnight at 4^{o}C. The capture antibody was removed and the plate washed six times in ELISA wash buffer. The plate was then blocked with 25µL of PBS containing 10% FBS and incubated at room temperature for 2 h to saturate non-specific binding sites. Blocking buffer was removed by flicking and titrating amounts of IFNγ standard (BD biosciences) were added in duplicate across the plate starting at 10ngml^{-1} final concentration in assay diluent (PBS + 10% FBS) and titrating down 2-fold each time until 10 titrations were made. Supernatant samples were added directly to the remaining wells and the plate was incubated overnight at 4^{o}C. The plate was washed six times in ELISA wash buffer before the addition of assay diluent 0.2% biotinylated anti-IFNγ detection antibody (BD biosciences) then allowed to incubate at room temperature for 2 h in the dark. The plate was washed eight times in ELISA wash buffer then assay diluent with 0.25% avidin-peroxidase (BD biosciences) was added to the plate and allowed to incubate at room temperature for 45 min in the dark. The plate was washed a final eight times with ELISA wash buffer before the addition of developing substrate containing 1mgmL^{-1} of O-Phenylenediamine Dihydrochloride (Sigma-Aldrich) and 0.4% H_2O_2. The ELISA plate was allowed to develop and then stopped by adding 2M H_2SO_4 when the top four standards became visible. Absorbance was read on a microplate reader at 450nm.

2.10 Serum cytokines

Serum was collected at the indicated times within the text according to 2.3.1.2. Cytokines were measured in the serum using a Bio-Plex Pro™ Mouse Cytokine 23-plex Assay (BioRad Laboratories, CA, USA). To generate the Bio-Plex standard, 128µL of the reconstituted standard was mixed in a tube containing 72µL of Bio-Plex sample diluent by vortexing. To generate a 4-fold dilutions series, 50µL was taken from the stock tube and mixed in a new tube containing 150µL of sample diluent by vortexing. This process was repeated until a total of eight dilutions was reached. For a full 96-well plate, the 10x coupled beads were vortexed for 30 seconds then diluted to 1x in Bio-Plex assay buffer by taking 575µL of beads mixing with 5175µL of Bio-Plex assay buffer. The filter plate

was first prewet with 100µL Bio-Plex assay buffer then 50µL of well vortexed 1x beads was added to each well. The plate was washed two times by adding 100µL of Bio-Plex wash buffer before adding 50µL of standards or samples (diluted 1:4 with assay diluent) to each well. The plate was sealed and placed on shaking incubator at 850rpm for 30 min, protected from the light. After 20 min of incubation, the 10x detection antibody was vortexed vigorously then diluted to 1x by taking 300µL of detection antibody and diluting in 2700µL of detection antibody diluent. After the 30 min incubation, the plate was washed three times with 100µL of wash buffer before the addition of 25µL of 1x detection antibody to each well. The plate was sealed and placed on shaking incubator at 850rpm for 30 min, protected from the light. After 20 min of incubation the 100x SA-PE was vortexed briefly then diluted to 1x by taking 60µL of SA-PE and diluting in 5940µL of assay buffer. The plate was washed three times with 100µL of wash buffer before the addition of 50µL of the 1x SA-PE to each well. The plate was sealed and placed on shaking incubator at 850rpm for 30 min, protected from the light. The plate was washed three times with 100µL of wash buffer then the beads were resuspended in 125µL of assay buffer. The plate was sealed and placed on shaking incubator at 850rpm for 30 seconds. To read the plate, the seal was removed and read on the Luminex 200 array reader (Merck Millipore, MA, USA) on low PMT, RP1 setting. Serum cytokine concentrations were determined by the in-built software (Bio-Plex manager – Luminex xPONENT) against commercial standards (Bio-Rad laboratories) and lower limit of detections were set according to the appropriate lot numbers.

2.11 Statistical analysis

For biological studies, statistical analysis was performed on GraphPad Prism version 8.1.1 software (CA, USA). When comparing two groups a t-test was performed. When comparing multiple samples greater than two, a one-way ANOVA with a Tukey's multiple comparisons test was carried out where $P<0.05$ was considered significant. When using two parameters with multiple groups, two-way ANOVA with a Tukey's multiple comparisons test was used where $P<0.05$ was considered significant.

Chapter 3: The chemical synthesis, stability and activity of MAIT cell prodrug agonists that access MR1 in recycling endosomes

3.1 Introduction

The MAIT cell activatory ligands 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU), 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OP-RE) and 5-(methyl-2-oxopropylideneamino)-6-D-ribitylaminouracil (5-MOP-RU), are pyrimidines formed endogenously, via the non-enzymatic condensation of α-dicarbonyl glycolysis products with the riboflavin precursor 5-amino-6-D-ribitylaminouracil (5-A-RU, structure shown in Fig 3.1, and labelled "**1**")[185]. Within the endoplasmic reticulum, these carbonyl species form a covalent Schiff base with lysine 43 (K43) within the A' pocket of MR1, stabilising the MR1-antigen complex and triggering the trafficking to the plasma membrane for presentation to MAIT cells[205]. After cell surface expression, MR1 is internalised and, like other MHC-I molecules, is either targeted for degradation or may enter the recycling pathway where antigen exchange may occur with endosomal MAIT antigens such as those derived from endocytosed bacteria[205,213].

Unfortunately, research into MAIT cell immunology can be confounded by the instability of the most commonly prepared agonists 5-OP-RU, 5-OE-RU and 5-MOP-RU. In aqueous solution at 15°C, 5-OP-RU has a half-life of 14 h (pH 6.8), 5-OE-RU 18 min and 5-MOP-RU 14 h both at pH 8[204,229]. For biological studies, 5-OP-RU is most commonly used and is typically prepared *in situ* by the addition of an excess of MG to 5-amino-6-ribitylamino uracil (5-A-RU, 1) immediately prior to use. However, formation of 5-OP-RU (and other analogues) have been shown to rapidly cyclise into lumazines rendering them dramatically less potent due to their inability to bind to MR1 efficiently[229]. *In vitro* studies have demonstrated that 5-A-RU itself can activate MAIT cells within human PBMCs even without added glyoxal or MG (albeit, less efficiently than 5-OP-RU)[204]. This suggests that eukaryotic cells may provide a sufficient concentration of endogenous aldehyde metabolites that condense with 5-A-RU to provide a functional MR1 ligand. This indicates that 5-A-RU could be used without prior addition with MG in order to study the effects of MAIT cell activation both *in vitro* and *in vivo*. This approach would bypass the need for the prior synthesis of 5-OP-RU, which is subject to cyclisation to lumazines. However, as has been noted anecdotally by various groups[230,231], 5-A-RU is itself rather unstable.

In this chapter, the stability of 5-A-RU was definitively characterised, including assessment of ability to drive MAIT cell activation, following its exposure to storage conditions that would typically be used in biological studies. It was then investigated whether stabilisation could be achieved by modification of the 5-amino group with a cathepsin-cleavable valine-citrulline-p-aminobenzyl carbamate (VC-PAB). This well-established chemical modification[232,233] would not only allow stabilisation of the labile parent molecule, but also enforce an additional processing step to release the 5-A-RU structure, which would only occur in the presence of the proteolytic activity of cathepsins, found in high concentrations within APCs[234,235]. It was hypothesised that this step may, in turn, enhance localization of 5-A-RU with MR1, and therefore improve MAIT cell activation. Overall, the intention was that this chemical modification would lead to the development of stable and potent MAIT agonist precursors that would improve the capacity to study MAIT cells and their contribution to immunity.

3.2 Aims

A significant challenge in MAIT cell research is that both the agonist 5-OP-RU, and the MAIT agonist precursor, 5-A-RU, are chemically unstable. At the outset of the studies described below, it was speculated that 5-A-RU was labile to auto-oxidation, which may lead to loss in biological activity. Therefore, the overall aim of this chapter was to establish the extent and nature of the instability of 5-A-RU and then to develop a prodrug form of 5-A-RU to overcome stability issues. It was anticipated that, when combined with cells *in vitro* or *in vivo*, sufficient MG would be available intracellularly to permit condensation to form the MAIT cell agonist 5-OP-RU. Given that such a prodrug would require enzymatic processing, it was also anticipated that the route to the presentation of 5-A-RU on MR1 might be altered. This possibility was also investigated.

Specific aims covered:

- Assessment of auto-oxidation of 5-A-RU generated by two synthesis methods, infer decomposition products and determine their capacity to activate MAIT cells *in vitro.*
- Generation of stable 5-A-RU prodrugs and comparison of activatory potential versus native 5-A-RU *in vitro* and *in vivo.*
- Determination of the mode of presentation of the prodrug versus 5-A-RU using antigen-presenting cells that have been genetically modified to alter distribution of the presenting molecule, MR1.

3.3 Results

3.3.1 Instability of 5-A-RU

It has been reported that 5-A-RU is unstable, decomposing to a "multitude of products"[236]. To investigate this formally, 5-A-RU was prepared for stability studies. To achieve this, 5-A-RU (Fig 3.1, labelled "**1**") was prepared by palladium (Pd)-catalysed hydrogenolysis of 5-nitrosouracil **2.** Preparations of 5-A-RU usually are highly concentrated and require dilution in PBS or water for biological application. Thus, to mimic conditions in which 5-A-RU would be used for experiments, a freshly prepared batch was diluted in water and gently agitated while exposed to air at room temperature at various lengths of time in order to generate any natural degradation species. To characterise the products of this aeration, liquid chromatography mass spectrometry (LCMS) was performed by Dr Regan J. Anderson (Ferrier Research Institute). All chemical synthesis and chemical characterisation studies were performed by R.J.A and will be specified accordingly within. Methodology for the chemical analysis of the compounds in this chapter can be found in Appendix F.

During this process, the various reaction mixtures were eluted through a reverse-phase silica column, separating chemical species based on their charge, which alters the time of elution. These products were then viewed as separate species on a chromatogram based on the time of elution from the column. Once separated, these chemical species were identified through mass spectrometry. Through this process, each elution peak was identified based on their mass (m) and charge (z) to give a mass to charge ratio (m/z) which infers directly to its molecular weight.

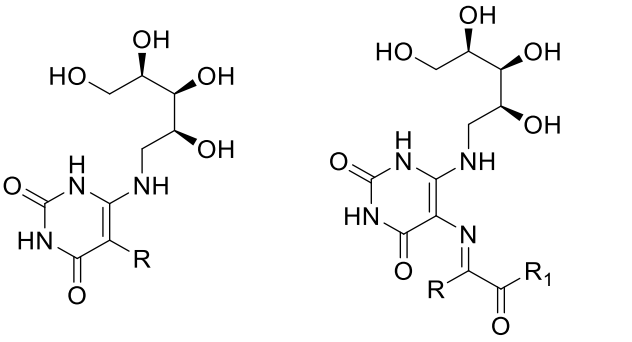
LCMS analysis (with care to avoid air exposure) showed one major peak, corresponding to 5-A-RU (Fig 3.2a, peak a, m/z: 277 = M+H). Exposure of this dilute sample (0.1 mg/mL) to air for 8 min led to the disappearance of this peak over 40 min, with the concomitant appearance of two new peaks b (m/z: 275) and c (m/z: 294). Peak b was short-lived, while peak c diminished more slowly and after one day was replaced by a new peak d with the same mass (m/z: 294). These results suggest the degradation of 5-A-RU proceeds with loss of dihydrogen to generate azaquinone species **3** (Fig 3.2b), corresponding to peak b. Hydrolysis gives intermediate **4** (not observed) which exists as

its hydrate **5** (peak c). A slow attack of water leads to the rearranged end-product **6** (peak d), analogous to the reported rearrangement of alloxan to alloxanic acid[237]

As an alternative synthetic route, generation of 5-A-RU reported for use within biological studies has been accomplished by sodium dithionite reduction of nitrosouracil **2**.[185] When a fresh sample of 5-A-RU prepared in this manner was briefly exposed to air, the formation of two earlier-eluting peaks was observed (Fig 3.3a, peaks e and f). As shown in Fig 3.3b, an initial dehydrogenation step was proposed to produce azaquinone **3**, which is rapidly attacked by sulfite (present as a by-product of dithionite oxidation) to give **7**. Sulfonate **7** may rearrange to give **8** and/or **9** which regenerates the uracil core in its original oxidation state.

To determine the potential of any downstream decomposition products to activate MAIT cells, the 5-A-RU preparations (from either method) were exposed to and allowed to stand for 5 h. In the case of the Pd-catalysed hydrogenolysis reaction product, samples were aged for either 5 h or 29 h, to give solutions enriched in peak c and peak d, respectively (Fig 3.2). These aged samples, together with fresh 5-A-RU samples prepared by both methods, were then assessed *in vitro* for their ability to activate MAIT cells in cultures of PBMCs. MAIT cells are highly represented in human PBMCs, and therefore easily identifiable by flow cytometry. Following exclusion of dead cells and B cells (the latter based on expression of CD19), MAIT cells were gated based on expression of their invariant TCRα variable fragment arrangement, Vα7.2, and high expression of the C-type lectin receptor CD161. During activation, T cells downregulate their TCR in response to interaction with their respective ligands. This process is typically followed by upregulation of 4-1BB (CD137), a member of the TNF receptor superfamily. It was therefore possible to assess MAIT cell activation using flow cytometry and a gating strategy that allowed for downregulation of the TCR, and then determining the percentage of $CD137^+$ MAIT cells in the cultures (Fig 3.4a). Upon stimulation of PBMC cultures with freshly prepared 5-A-RU for 18 h, MAIT cells readily down regulated their TCR and upregulated CD137. Indeed, 5-A-RU prepared by either method activated MAIT cells in a dose dependent manner to a similar degree (Fig 3.4b, c). This confirms previous findings[227] that in the absence of exogenously added MG the intracellular concentration of dicarbonyls is sufficiently high to react with 5-A-RU to generate MR1 binding ligands such as 5-OP-RU or 5-OP-RE. Weak MAIT cell activation was observed for the aged 5-

A-RU sample obtained from dithionite reduction (Fig 3.4b), while neither of the samples obtained from the hydrogenolysis reaction activated MAIT cells (Fig 3.4c). Combined with the observation that 5-A-RU is similarly active when carefully prepared by either method, this effectively rules out the downstream oxidised products 3 - 9 as contributors to MAIT cell activation.



R = NH_2 (**1** = 5-A-RU)
R = NO (**2**)

5-OE-RU, R = R_1 = H
5-OP-RU, R = H, R_1 = Me
5-MOP-RU, R = R_1 = Me

Figure 3.1. Chemical structure of MAIT cell agonists.

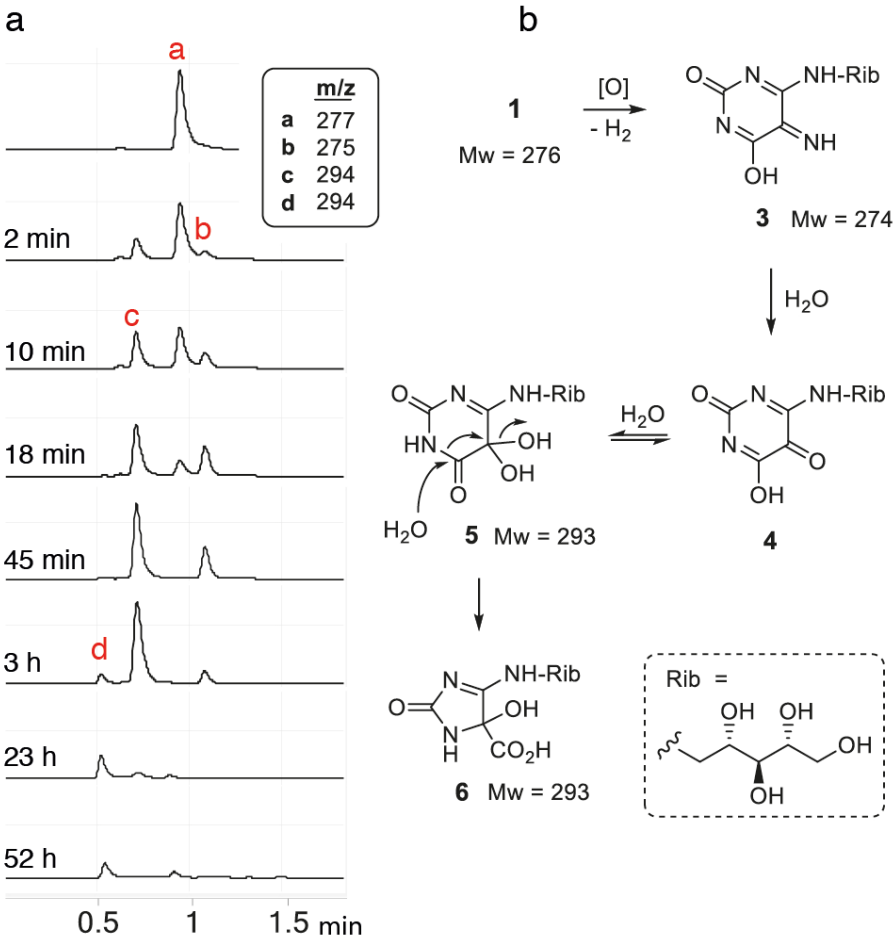


Figure 3.2. Auto-oxidation of 5-A-RU prepared by Pd/H2 catalysis. (a) LCMS analysis (254 nm) of 5-A-RU (1) freshly prepared using Pd/H2 (top trace), and 5-A-RU exposed to air (8 min) by gentle agitation followed by standing for the indicated times (see supporting information for details); B. Inferred oxidative decomposition of 5-A-RU (1) in dilute aqueous solution. Other tautomeric forms may be considered. Experiment performed by R.J.A.

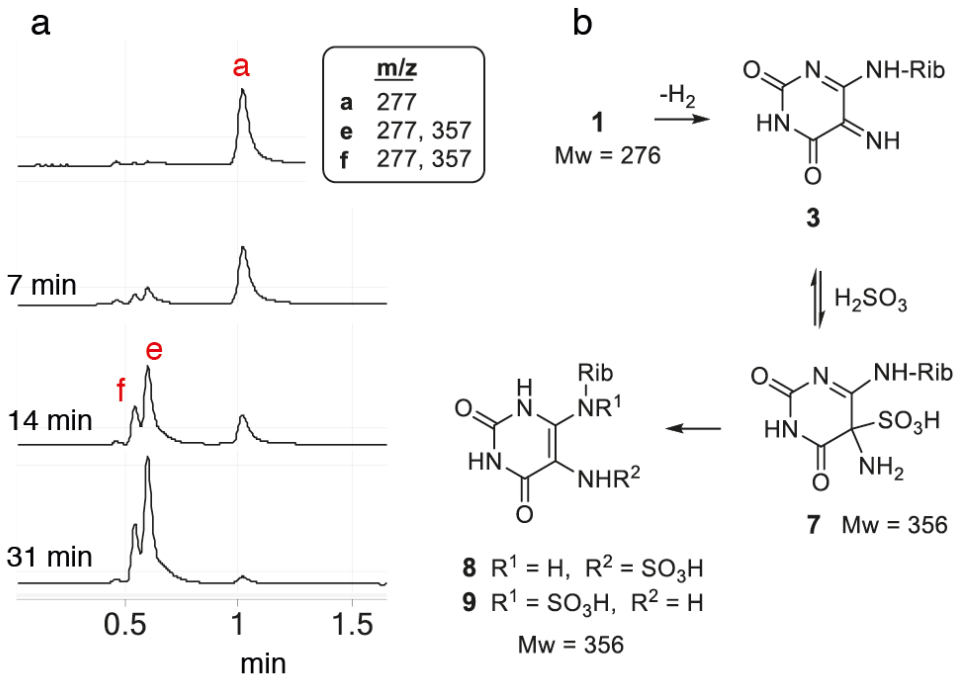


Figure 3.3 Auto-oxidation of 5-A-RU prepared by sodium dithionite reduction. (a) LCMS analysis (254 nm) of 5-A-RU (1) freshly prepared using sodium dithionite (top trace), and dithionite-produced 5-A-RU exposed to air (3 min) followed by standing for the indicated times.(b) Inferred oxidative decomposition of 5-A-RU (1) in aqueous solution containing sulfite. Experiment performed by R.J.A.

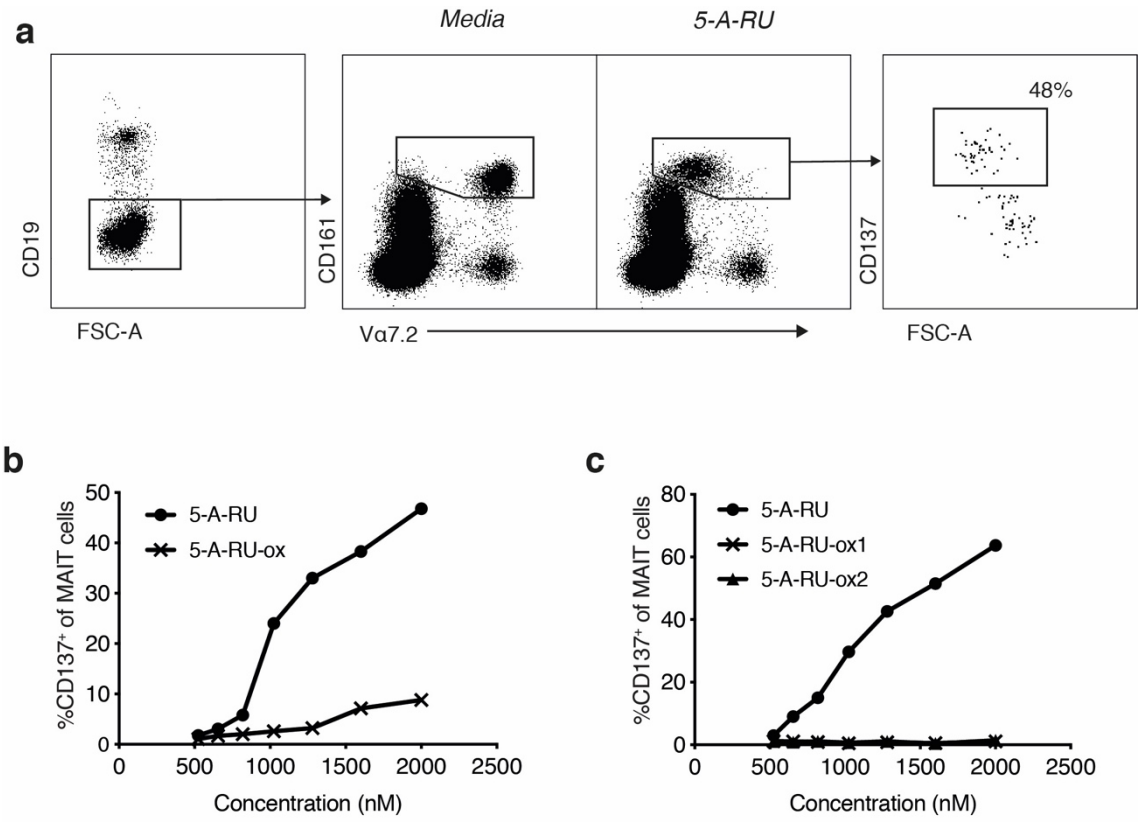


Figure 3.4. Oxidized 5-A-RU preparations fail to activate MAIT cells. (a) Gating strategy for MAIT cells, defined as CD19^{-} CD161^{hi} Vα7.2^{+} cells, depicting TCR downregulation during activation with 5-A-RU. Human PBMCs from a single donor were incubated for 18 h with titrating amounts of the indicated 5-A-RU preparations (1x10^{6} cells per preparation). (b) 5-A-RU was prepared from the reduction of nitrosouracil 2 with sodium dithionite; 5-A-RU-ox was prepared by the same method but aerated for 5 h (c) 5-A-RU was prepared from the reduction of nitrosouracil 2 by Pd-catalysed hydrogenolysis; 5-A-RU-ox1 and -ox2 were prepared by the same method but aerated for 5 h and 19 h, respectively. Activation of MAIT cells is depicted as percentage of MAIT cells positive for CD137 at the end of the culture period. Data representative of two separate experiments.

3.3.2 Synthesis of 5-A-RU prodrugs

The propensity of 5-A-RU to autoxidation necessitated careful handling and storage for biological studies. Even with such precautions, degradation could not be avoided in our experience, especially in dilute solutions in which oxygen diffusion is a more significant issue. Additionally, *in vivo*, dissolved oxygen in blood or tissues may lead to significant losses in potency. Therefore, the ability to generate a stable MAIT cell agonist without specialist chemistry skills would be a welcome advance and potentially facilitate more consistent results. The uracil core of 5-A-RU is electron-rich, contributed partly by the 5-amino group, making it labile to attack by electrophilic oxygen molecules, leading to oxidation. It was posited that generation of a prodrug by modification of the 5-amino group with a (cleavable) electron-withdrawing group would temper the electron-rich uracil core, stabilising 5-A-RU against oxidation. Upon enzymatic cleavage of the prodrug, 5-A-RU may be released intracellularly. Here, 5-A-RU could combine with endogenous dicarbonyls (that are present as metabolic products in higher concentrations inside cells) to provide a functional MR1 ligand with agonism for MAIT cells.

Synthesis of compound **10** was, therefore, the primary focus to test this hypothesis (Fig 3.5). The VC-PAB linker present in **10** is widely used as a cleavable linker in antibody-drug conjugates[232]. Cleavage by cathepsin B at the citrulline carboxyl side, gives an aniline intermediate which spontaneously collapses with loss of CO_2 to release the drug-amine (in this case, 5-A-RU)[233]. Cathepsins and other proteases are highly expressed in APCs and are key enzymes involved in protein degradation as well as processing long antigenic peptides into oligomeric fragments to be loaded onto MHC molecules[234,235]. In this way, chemical conjugation of 5-A-RU to a VC-PAB linker should mediate the release of 5-A-RU preferentially within APCs, and also potentially protect the reactive 5-amino group of 5-A-RU until it is in close proximity to MR1 molecules. To investigate the importance of cathepsin-mediated cleavage of the linker in **10**, a stereoisomer was also prepared, which contains *D*-citrulline at the cleavage position (compound **11**). The use of the stereoisomer would act as a retardant against cleavage by cathepsin by disallowing enzymatic access. Thus, evaluation of the capacity of compound **11** to stimulate MAIT cells would test whether release of 5-A-RU was indeed mediated by cathepsin cleavage at the VC sequence. A third prodrug was developed as a simple carbamate derivative, **12** (Fig 3.5). This analogue lacks any cleavage site for cathepsin and simply acts as a

stabilising terminal group, which was assessed to again emphasise the importance of an intact and accessible VC sequence.

To examine the stability of these modified 5-A-RU-derivatives, aqueous solutions of **12** and **10** were exposed to air and agitated on a mechanical shaker at ambient temperature for 24 h. The samples were subsequently analysed by LCMS at various time points. As shown in Fig 3.6, minimal degradation was observed in both samples over 21 d, demonstrating that, even with deliberate over-exposure to air, these modified 5-A-RU-derivatives were much more stable than the parent molecule, and that no special care in handling these compounds is required.

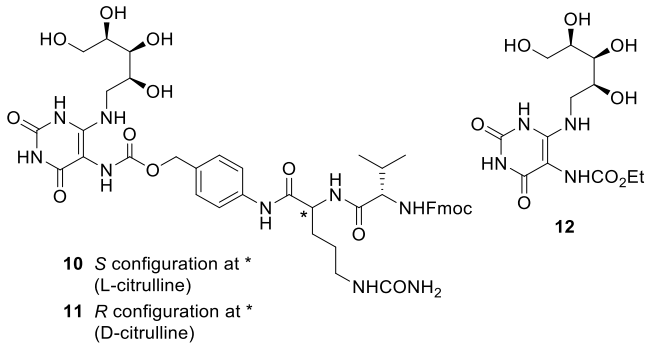


Figure 3.5. Chemical structure of 5-A-RU prodrugs.

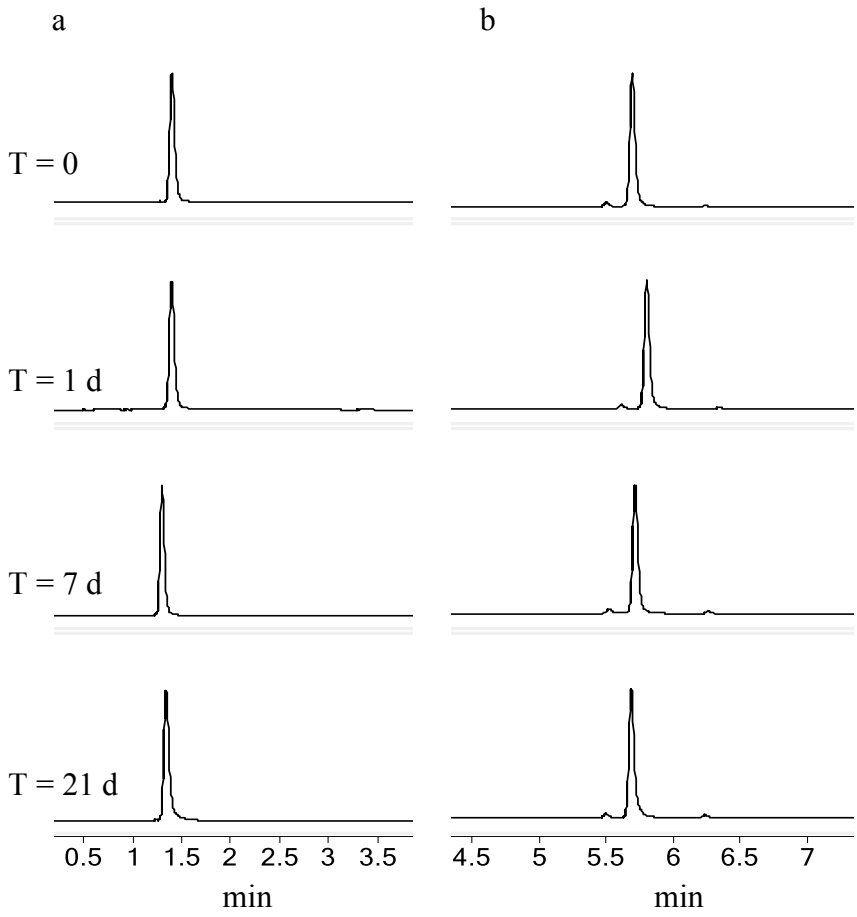


Figure 3.6. Stability of 5-A-RU prodrugs. Stability as determined by LCMS of (a) 12 in water, and (b) 10 in 1:4 DMSO:water at room temperature, including 24 h exposure to air. Experiment performed by R.J.A.

3.3.3 5-A-RU prodrugs can activate human MAIT cells.

To compare the ability of 5-A-RU and its prodrugs to activate MAIT cells, the compounds were diluted in PBS with or without MG. In the case of 5-A-RU itself, it was anticipated that this would allow condensation to 5-OP-RU before incubation with human PBMCs (although, as noted in the introduction, this is not a practical process to give reproducible biological results, due the instability issues described here). With the prodrug design, it was anticipated that preincubation with MG was unnecessary, as MG would be supplied intracellularly. Significantly, prodrug **10** started activating MAIT cells at ~10-fold lower doses than 5-A-RU without MG (Fig 3.7a), highlighting the increased potency of the stabilised MAIT agonist precursor. As expected, the presence of MG did not alter the activity of **10** confirming the protection of the 5-amino group prior to intracellular cleavage of the linker. After preincubation with exogenous MG, 5-A-RU was expected to be converted to 5-OP-RU, which in turn was more potent than **10**, perhaps suggesting that supply of intracellular dicarbonyls ultimately limited the activity of **10**. Compounds **11** and **12** were assessed similarly alongside **10**. The simple carbamate **12**, lacking a cleavage sequence, did not show any activity, even at the highest concentration examined (Fig 3.7b). As the carbamate structures in **12** and **10** are electronically and sterically similar, these results suggest that enzymatic cleavage is important in releasing an active agonist (in the case of **10**). This was further supported by the observation that replacing *L*-citrulline with *D*-citrulline in the cathepsin cleavage site (compound **11**) resulted in much reduced activity. It is likely that the weak activity observed for this epimer was due to residual natural *L,L*-isomer (i.e., **10**) contaminating the material (~0.6% as determined by HPLC analysis, data not shown).

Although a range of proteases are found in the late endosomes and lysosomes and could be involved in processing of the Val-Cit dipeptide linkers, cathepsin B is believed to play the most prominent role[233]. Therefore, *in vitro* susceptibility to cathepsin B cleavage may serve as an indicator of a prodrug or conjugate's *in vivo* propensity to cleavage. Prodrugs **10** and **11** were incubated with human liver cathepsin B and progression of cleavage was followed by LCMS. After 4 h, around 75% of **10** had been consumed while in the same time frame **11** remained intact (Fig 3.8). No cleavage of **10** was observed in the absence of enzyme (data not shown). These results corroborate those from the MAIT cell activation experiments and together point to the importance of cathepsin B or functionally similar processing enzymes in the generation of an active species.

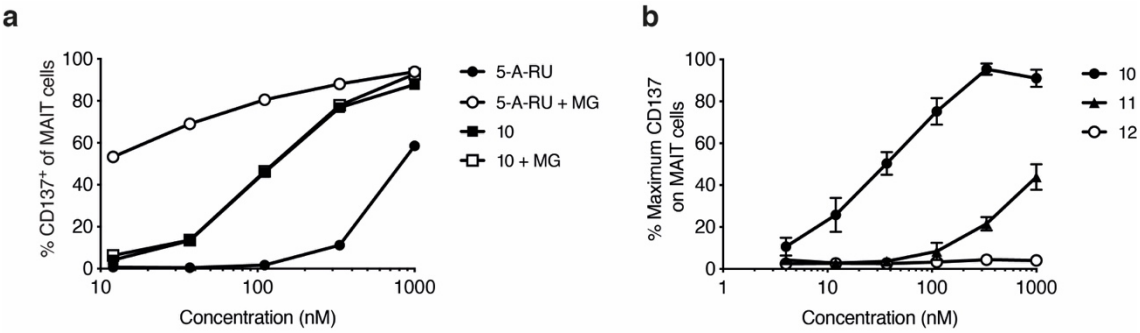


Figure 3.7 Activation of human MAIT cells with 5-A-RU prodrugs. Human PBMCs were incubated for 18 h with different concentrations of the indicated compounds, and then the percentage of activated MAIT cells in the cultures determined by flow cytometry. (a) 5-A-RU, 5-A-RU + MG, 10 or 10 + MG; (b) 5-A-RU carbamate derivatives 10, 11 and 12. Activation of MAIT cells is depicted as percentage of MAIT cells positive for CD137 at the end of the culture period combined from three separate donors. MAIT cells were identified as $CD19^{-}CD3^{+}V\alpha7.2^{+}CD161^{hi}$ cells.

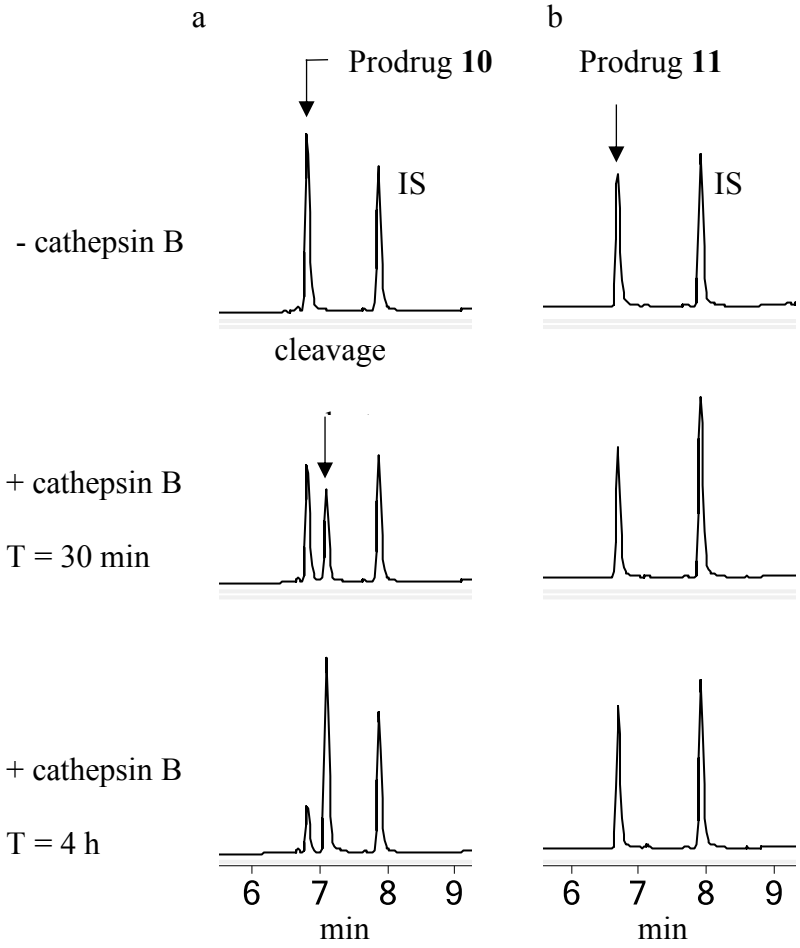


Figure 3.8. Cathepsin mediated cleavage of 5-A-RU prodrugs. LCMS analysis of in vitro cathepsin B-mediated cleavage of (A) 10, and (B) 11. Samples were incubated at 37 °C with or without cathepsin B and analysed after 30 min and 4 h. Detection is by MS [combined extracted ion chromatograms for prodrug, expected cleavage product and internal standard (IS)]. Experiment performed by R.J.A.

3.3.4 5-A-RU prodrugs access the recycling endosome

Similar to MHC class Ia molecules, MR1 is predominantly loaded with soluble antigens within the ER. However, after presentation on the cell surface, MR1 is internalised, and a proportion of molecules are able to sample and exchange antigens within the recycling endosome[205,214]. Interestingly, it has been shown that MAIT cell activation by mycobacterial supernatant containing soluble MAIT antigens could be blocked by pre-saturation with the soluble MR1 antagonist, acetyl-6-formylpterin (Ac-6-FP), whereas stimulation with whole mycobacteria was not affected by blocking with Ac-6-FP[205]. This suggests separate loading compartments, with MAIT antigens derived from intracellular infection potentially entering a distinct pathway that does not involve the ER, and may therefore require access to MR1 in recycling endosomes that fuse with the incoming phagocytic vesicle.

As the 5-A-RU prodrug **10** requires protease cleavage before the release of 5-A-RU, and these enzymes are rich in endolysosomal compartments, it was possible that the 5-A-RU released from **10** can similarly access MR1 molecules from the recycling pathway. To test this hypothesis, PBMCs were pre-incubated with Ac-6-FP prior to incubation with 5-A-RU or **10**. As expected, activation of MAIT cells with 5-A-RU was inhibited by Ac-6-FP, indicating presentation of 5-A-RU was effectively blocked. In contrast, the presentation of MAIT cells agonists from the processing of **10** was largely unaffected, suggesting that loading of the released ligand onto MR1 occurs predominantly in a different compartment (Fig 3.9a). To test the requirement of acidified compartments such as recycling endosomes in the release of 5-A-RU molecules from **10**, cells from the immortalised monocyte-like cell line, THP-1, were incubated with bafilomycin A1, an inhibitor of the vacuolar ATPases required to lower the pH, before being exposed to 5-A-RU or **10** and then incubated with human MAIT cells. To increase the sensitivity of the assay, MAIT cells were purified from PBMCs by fluorescence-activated cell sorting (FACS) before analysis was conducted. The readout of activity was release of IFN-γ into the supernatant once MAIT cells were stimulated, which was determined by enzyme-linked immunosorbent assay (ELISA). As hypothesised, inhibition of endosomal acidification with bafilomycin abrogated MAIT cell activation by **10**, but not 5-A-RU. In fact, bafilomycin A1 actually increased presentation of 5-A-RU, an outcome that has been previously reported[238] (Figure 3.9b).

To examine presentation of **10** further, studies were conducted with MR1-KO THP-1 cells[226] that were then modified to overexpress either full-length MR1, or MR1 with a glycosylphosphatidylinositol (GPI) linker that covalently links it to the outer leaflet of the plasma membrane to prevent internalisation into recycling endosomes. While the presence of the GPI anchor did not affect the ability of 5-A-RU to be presented to purified human MAIT cells (again as defined by IFN-γ release), the presentation from **10** was substantially reduced (Fig 3.9c and d). It is therefore highly likely that MR1-loading from the prodrug occurs through access to MR1 in recycling endosomes. In addition, the agonist released from **10** required bonding to a K43 on MR1, located within the binding groove of MR1, which is typical of the known derivatives of 5-A-RU, as no MAIT cell activation could be induced when incubated with THP-1 cells overexpressing MR1 with an alanine substitution at position 43 (Fig 3.9c).

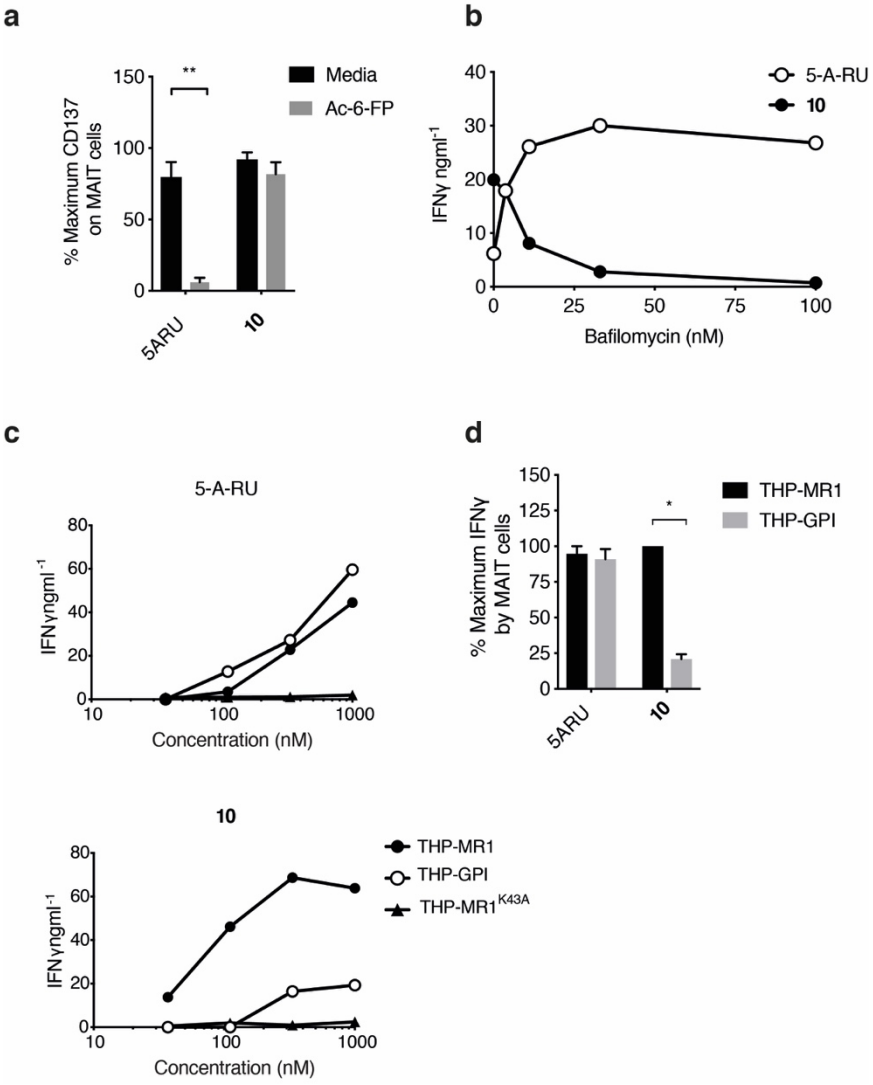


Figure 3.9. 5-A-RU prodrug is processed and presented via the recycling endosome. (a) Human PBMCs were incubated with Ac-6-FP or media for 2 h prior to addition of 5-A-RU or compound 10. Cells were incubated for 18 h and MAIT cell activation was assessed via flow cytometry. (b) The modified THP-1 cells were pre-incubated with increasing amounts of bafilomycin A1 for 2 h, 5-A-RU or 10 was added and then cultured for a further 18 h before co-culture with human MAIT cells. Supernatant was taken after 24 h and IFNγ was measured. Data representative of two separate experiments. (c) Purified human MAIT cells were incubated with either THP-MR1, THP-GPI or THP-K43A cell lines loaded with titrating amounts of 5-A-RU or 10. After 24 h, IFNγ production by MAIT cells was measured in the supernatant. Data representative of three separate experiments. (d) Combined data from three separate human donor MAIT cells co-cultured with THP-MR1 or THP-MR1 cells lines incubated with equivalent 5-A-RU or 10. Activation of MAIT cells for (a) is depicted as percentage of MAIT cells positive for CD137 at the end of the culture period. MAIT cells were identified as $CD19^{-}CD3^{+}V\alpha7.2^{+}CD161^{hi}$ cells. Graphs are depicted with the mean ± SEM. Statistical significance was determined by a two-way ANOVA with a Tukey's multiple comparisons test.

3.3.5 5-A-RU prodrug activates MAIT cells *in vivo*

To determine whether prodrug **10** could activate MAIT cells *in vivo*, and perhaps show a benefit over 5-A-RU, studies were conducted in mice, which as noted in the Introduction, harbour MAIT cells with similar TCR structures, although cell numbers are reduced compared to humans. The compounds were administered intravenously, and assessment of MAIT cell activation was conducted in lungs, where measurable populations of MAIT cells are known to exist[23]. After 18 h, activation of MAIT cells in the lungs of mice was assessed by flow cytometry using fluorescent 5-OP-RU-loaded MR1 tetramers and expression of the TCR β chain to identify the MAIT cell population (Appendix B). As shown (Fig 3.10), both 5-A-RU and **10** were capable of activating MAIT cells at a high dose (180 nmol) as indicated by expression of the early activation marker CD69, and the IL-2 receptor CD25. However, at a lower dose of 5 nmol, 5-A-RU did not induce any obvious activation of MAIT cells, while, compared to untreated controls, prodrug **10** increased expression of these activation markers, indicative of *in vivo* activation. Thus, not only does the prodrug have the advantage of being significantly more stable than 5-A-RU, it is also a more potent agonist.

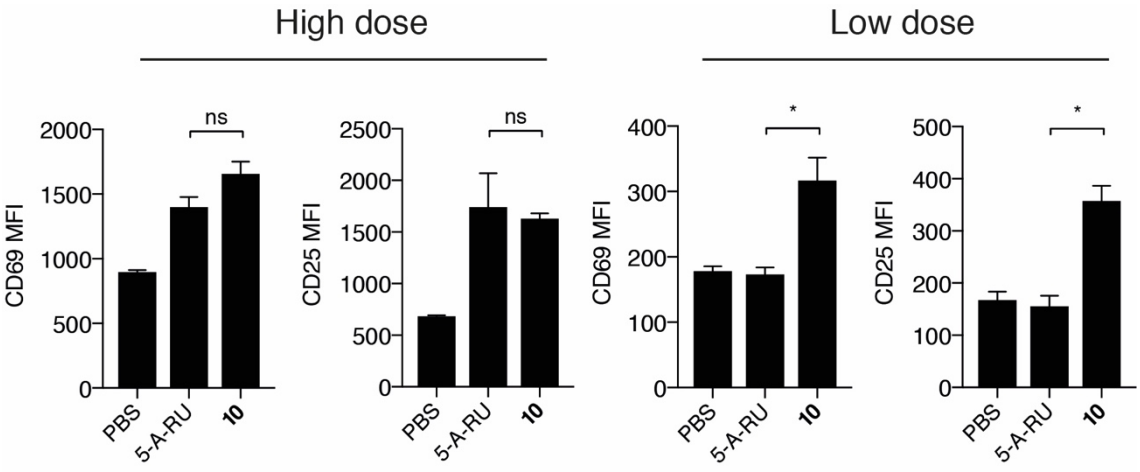


Figure 3.10. 5-A-RU prodrug induces activation of murine MAIT cells *in vivo.* Mice (n=5) were administered intravenously PBS, 180 nmol (high dose) or 5nmol (low dose) 5-A-RU or 10. After 18 h, lungs were harvested and stained for flow cytometry. MAIT cells were defined as $CD45^{+}B220^{-}CD11b^{-}$ and 5-OP-RU $tetramer^{+}$. Median fluorescence index (MFI) was calculated for the markers CD69 and CD25 on MAIT cells. Graphs are depicted with the mean ± SEM. Statistical significance was determined by a One-Way ANOVA with a Tukey's multiple comparisons test.

3.4 Discussion

The instability of the MAIT cell agonist precursor, 5-A-RU, has been reported anecdotally previously[230,231], but no formal stability study had been published at the outset of this study. Given that this material is used many biological studies, it was necessary to characterise the extent and nature of degradation when exposed to oxygen, which could occur in the storage solutions used by many researchers. In this chapter, it was shown that, indeed, when 5-A-RU was diluted in aqueous solutions, it was immediately susceptible to oxidation. Auto-oxidation was apparent in 5-A-RU samples derived from synthesis via both sodium dithionite reduction or Pd/H_2 hydrogenolysis of nitrouracil, with the appearance of alternative eluting peaks seen by LCMS as early as a few minutes. This instability was perhaps unsurprising since the lone pair of electrons located on the 5-amino nitrogen make it particularly susceptible to electrophilic attack. This chapter provides the first theoretical schematic of the species formed after 5-A-RU is exposed to air. Oxidation of the uracil ring at the 5-amino position followed by deamination (Fig 3.2b) or *N*-sulfation (Fig 3.3b) is of particular importance as this amine is the site of condensation with dicarbonyls which converts 5-A-RU into 5-OP/OE/MOP-RU. The newly formed formyl group at this position is responsible in the formation of a Schiff base with the K43 residue on MR1, inducing conformational stabilisation, translocation to the cell surface and consequently TCR-mediated activation of MAIT cells[204]. Therefore, oxidation of the parent molecule 5-A-RU at the 5-amino position would obstruct dicarbonyl condensation and thus preclude bond formation with K43 on MR1, impeding MAIT cell activation. These results emphasise the limitations of using 5-A-RU in biological studies or drug development, with the high susceptibility to oxidation likely to pose a significant problem with respect to the reproducibility of experimental procedures or treatments.

To address this problem, the instability of 5-A-RU was overcome by the synthesis of a stable prodrug that can be cleaved intracellularly to provide active MAIT cell agonist precursor(s). Importantly, when added to cultures containing MAIT cells, prodrug **10** induced MAIT cell activation at lower doses than 5-A-RU alone, emphasising the increased potency of the stabilised MAIT agonist precursor. While this may be explained to some extent by the enhanced stability, it is also possible that the altered mode of presentation of the prodrug is a contributing factor. Unlike 5-A-RU, prodrug **10** was still

able to induce MAIT cell activation in the presence of Ac-6-FP, which blocks 5-A-RU mediated activation and therefore suggests an alternative loading mechanism. However, the capacity of prodrug **10** to stimulate MAIT cell activity was blocked by inhibiting acidification of endosomes with bafilomycin A1, presumably because cathepsins within these vesicles require low pH to drive enzymatic cleavage at the VC sequence in the prodrug linker. Previous studies have highlighted the importance of acidified endosomes in the presentation of MAIT cell antigens, specifically those derived from whole bacteria. *In vitro* studies show reduced activation of MAIT cells when they were co-incubated with *Escherichia coli*-loaded bone marrow-derived DCs that had been treated with chloroquine, another inhibitor of endosomal acidification[199]. Similarly, another study showed that THP-1 cells loaded with whole bacteria, but not bacterial supernatant, had reduced capacity to activate MAIT cells when treated with bafilomycin A1[238]. It is unknown yet if agonists from the bacteria are loaded onto endosomal MR1 molecules, or if the localisation of bacteria within these compartments provides a highly concentrated pool of agonists that then traffic to the ER for presentation. Interestingly, 5-A-RU actually induced higher levels of MAIT cell activity in the presence of increasing bafilomycin A1 dose. One explanation for this is that 5-A-RU is becoming sequestered and trapped within lysosomes inhibiting access to MR1 molecules and thus lowering MAIT cell activation. Therefore, when lysosomal acidification is neutralized by bafilomycin, 5-A-RU may be released back into the cytosol in order to access MR1. As 5-A-RU contains a weak amine group, it is possible that it exhibits some degree of tropism for lysosomes. In this manner, the net neutrality and low molecular weight of 5-A-RU would support passive diffusion down its concentration gradient through the lipid bilayer of lysosomes. The free amine base on 5-A-RU would then become protonated due to the low pH of the endosome. This protonation creates a concentration gradient where the levels of native 5-A-RU within the cytosol now exceeds 5-A-RU within the lysosome therefore driving the accumulation of more 5-A-RU into the lysosome by diffusion in a process known as pH partitioning[239]. Protonation of the 5-amino group would also result in electrostatic repulsion of 5-A-RU molecules from the hydrophobic lipid bilayer; thus, inhibiting translocation out of the lysosome towards ER-localised MR1. Bafilomycin is an inhibitor of V-ATPases. Therefore, incubation with cells would result in increased pH levels within the lysosomal compartment. This neutralisation would then facilitate escape and lead to the increased availability of 5-A-RU and therefore increased levels of MAIT cell activation. As an additional explanation, 5-OP-RU molecules have been reported to have substantially

lower half-life at lower pHs going from 12 h at pH 6.8 to 44 min at pH 5.4[229]. Matured endosomes typically reach a pH of ~5. Therefore, neutralisation of endosomes may simply promote stabilisation of 5-A-RU once it has condensed with dicarbonyls.

While the experiments with bafilomycin A1 shows the requirement of acidified endosomes for the presentation of MAIT ligands derived from the prodrug, bafilomycin A1 is known to have some additional effects on cells at high concentrations such as cell cycle arrest and Bax-dependent apoptosis[240,241]. Therefore, the use of THP-1 cells that express MR1 with a GPI anchor allowed for the direct assessment on the presentation of prodrug **10** vs 5-A-RU when MR1 could not internalise into recycling endosomes without affecting other cellular processes. Indeed, the presentation of agonists derived from prodrug **10** was drastically inhibited in THP-GPI cells compared to THP-MR1, whereas 5-A-RU was not. This study, therefore, provides definitive evidence that MAIT agonists can indeed be loaded onto recycling MR1 molecules which occurs after internalisation of MR1 from the cell surface, and that by using a prodrug, this can be encouraged to take place in the absence of the requirement for the uptake of cellular bacterial material. It remains to be established whether presentation via MR1 in recycling endosomal molecules, as opposed to the ER (the predominant route for 5-A-RU and 5-OP-RU), leads to qualitatively different MAIT cell responses. A simple prodrug like **10** could be used to investigate this possibility further. The fact that **10** would be presented without additional bacteria-derived signals, such as TLR agonists, would enable the MR1-antigen presentation axis to be studied in isolation.

The overarching aim of this thesis, developed more in later chapters, was to determine the ability of MAIT cells to adjuvant T cell responses through 'helper' signals such as those able to be provided by $CD4^+$ T and NKT cells. Before initiating the studies presented here, *in vitro* experiments conducted by our colleagues had shown that once activated in the presence of 5-A-RU, human MAIT cells can mature APCs in an MR1 and CD40L dependent manner[29]. This important observation suggests that MAIT cells can potentially control adaptive responses. The studies presented in this chapter indicate that *in vivo* analyses to explore this further might proceed more efficiently with a stabilized compound. However, prodrug **10** did not activate MAIT cells to the same magnitude as 5-A-RU that had been reacted with exogenous MG to form 5-OP-RU prior to incubation. The utility of the prodrug may, therefore, be limited by the availability of

MG (or other relevant dicarbonyl compounds) to form relevant agonists. Whether this difference in activation will be mirrored in the ability of prodrug **10** to induce MAIT-dependent activation of DCs compared to 5-A-RU + MG will be explored in the next chapter.

A significant advantage of the prodrug design is that it could allow for further chemical modifications. In light of our experience in designing improved vaccines through ligation of antigenic peptides to immune adjuvants, such as to the NKT cell agonists α-GalCer[242,243], one possibility was to ligate peptides that will be released with 5-A-RU within the same APC upon cleavage by intracellular cathepsins. This is explored in the next chapter. Other possibilities, not explored in this thesis, include conjugation to TLR agonists or antibodies to target specific cell subtypes; each is described briefly below.

TLR-signalling has been shown to be an important auxiliary signal in both MR1 presentation and MAIT cell activation. Co-culture with the TLR9 agonist, CpG, induced the upregulation of MR1 on B cells[244,245]. In another study, conducted *in vivo*, intranasal administration of the TLR2 agonist Pam2Cys with 5-OP-RU induces enhanced expansion of MAIT cells in the lung and results in enhanced protection against pulmonary infection of mice with *Legionella longbeachae*[246,247]. These studies provide grounds to conjugate 5-A-RU with TLR agonists, as co-localising these molecules within the same APC (with subsequent cleavage) could subsequently boost MAIT cell activation. One limitation of this approach is the localisation of many TLR agonists on the cell surface, namely TLR1/2, TLR3, TLR4, TLR5 and TLR6[2,3]. A 5-A-RU TLR conjugate, as demonstrated in this chapter, would require intracellular processing to release both molecules. Therefore, the released TLR agonist would fail to interact with its receptor located on the cell surface. In contrast, TLR7/8 or TLR9 which recognise microbial nucleic acid motifs, are internally expressed, therefore agonists for these receptors would be a viable option for conjugation with 5-A-RU.

Adding a targeting motif to a prodrug form of 5-A-RU could also allow for selective targeting of 5-A-RU to specific cell subtypes. This approach has been successful in targeting antigens to cells expressing Clec9A, where conjugation of Clec9A-specific antibodies with weakly immunogenic influenza or enterovirus antigens resulted in trafficking of the antigens to Clec9A$^+$ DCs (cDC1 cells). As these cells have enhanced

capacity for cross-presentation, this led to induction of improved $CD8^+$ T cell responses and was also shown to improve antibody affinity and titers[248]. In a similar approach, 5-A-RU prodrugs could be conjugated to targeting antibodies towards specific APC subsets. In this way, interactions with MAIT cells between specific APC subsets could be studied *in vivo,* such as their ability to modulate their activation state through cytokines or cell-cell contact. This approach is highly relevant as the interactions between MAIT cells and different APC subsets is still unclear.

In summary, this chapter provides novel characterisation on the instability of 5-A-RU and its mode of auto-oxidation. The instability could be overcome by tempering the oxygen-sensitive 5-amino group with a cathepsin-labile linker group which not only completely stabilised 5-A-RU but also increased its potency in *in vitro* and *in vivo* settings. Analysis of the presentation of 5-A-RU prodrugs also supported previous evidence that 5-A-RU may be presented in a non-ER manner via the recycling endosome; as such the prodrugs could be used as novel tools for the exploration of MR1 presentation pathways. Moreover, this prodrug design has the potential for a multitude of further modifications to explore the field of MR1 and MAIT cell biology.

Chapter 4: Investigation on the adjuvant activity of 5-A-RU and vaccine potential of 5-A-RU-peptide conjugates

4.1 Introduction

MAIT cells are well documented as potent anti-bacterial effector T cells, exhibiting specific cytotoxicity towards bacterially infected cells[199,217]. Moreover, mice deficient for MR1 and therefore MAIT cells display defects in their ability to control models of pulmonary infection by *Klebsiella pneumoniae* and *Francisella tularensis*[26,187]. Though current studies highlight the necessity of MAIT cells in these bacterial responses, the mechanism by which MAIT cells are able to confer protection *in vivo* is poorly understood. A major mechanism proposed by current literature is that MAIT cells may directly contribute to the clearance of pathogens via lysis of infected cells. Additionally, there is also some evidence to suggest that MAIT cells may also be influencing adaptive responses in these models. Compared to WT, $MR1^{-/-}$ mice infected with *F. tularensis* were significantly impaired in their ability to recruit IFNγ producing $CD4^+$ and $CD8^+$ T cells to the lung[26]. Moreover, *in vitro* and *in vivo* activated MAIT cells can produce pro-inflammatory cytokines such as IFNγ, TNFα and IL-17, which are known to contribute to the development of adaptive responses[23,25,216]. Together these observations indicate that MAIT cells may indeed be able to influence adaptive immune responses, i.e. T and B cells. A recent study has established that MAIT cells can influence B cell activation directly. In a murine model for systemic lupus erythematosus, MAIT cells were required for germinal centre formation and the expansion of activated $CD4^+$ T cells[189]. Moreover, antibody production by B cells *in vitro* was dependent on CD40/CD40L signalling provided by MAIT cells. This study indicates that MAIT cells are indeed able to influence adaptive responses *in vivo* and suggests that activation of MAIT cells could be combined with antigens to drive antigen-specific T and B cell responses.

Currently, there is a high need for safe and efficacious adjuvants to augment vaccination strategies against microbial infection or against tumours[249]. Due to their abundance in humans, MAIT cells are an appealing target for the use as a cellular adjuvant in this role. T cells, and in particular $CD8^+$ T cells, are critical mediators of protection across a plethora of pathologies. Tumour infiltrating $CD8^+$ T cells are highly associated with positive outcomes in cancer, due to their ability to readily penetrate tissues and exhibit targeted destruction of malignant cells[135–137]. MAIT cells are also highly enriched within the mucosa. Therefore they are attractive targets as mucosal adjuvants for diseases that infect via mucosal interfaces such as HIV and *Mycobacterium tuberculosis* where the

generation of antigen-specific $CD8^+$ T cells have been shown to confer protection from disease progression[250–252].

As mediators of the adaptive immune response, DCs provide essential signalling towards T cells to prime their effector functionality. In order to study the ability of MAIT cells to modulate such $CD8^+$ T cell responses, the interactions between MAIT cells and DCs must first be properly defined. There is some evidence to suggest that MAIT cells can indeed influence the activation state of DCs. In an *in vivo* model of pulmonary infection with *F. tularensis*, differentiation of inflammatory monocytes within the lung was shown to require GM-CSF production dependent on MAIT cells[27]. As GM-CSF is an important developmental cytokine for DCs[253,254], this is amongst the first evidence that they possess some ability to modulate DC responses. When human MAIT cells are co-cultured with immature monocytes and 5-OP-RU, they are able to induce CD40L-dependent maturation of the monocytes, as well as induce the release of bioactive IL-12[29]. This suggests that MAIT cells can enhance DC function, and could therefore potentially be exploited as cellular adjuvants to stimulate adaptive T cell responses. Such a cellular adjuvant role has been described for NKT cells, where *in vivo* administration of the potent NKT cell agonist, α-GalCer, leads to CD40L-dependent activation of DCs. When such administration is combined with protein or peptide antigens, this leads to expansion of antigen-specific $CD4^+$ and $CD8^+$ T cells[19,20,255]. This potent adjuvant activity occurs in the absence of any other stimulation, such as through PRRs, and the T cell responses induced have been shown to be effective in driving antigen-specific immune responses in different disease models including malaria, influenza and cancer[178–181]. In line with observations *in vivo*, preliminary results in a human phase I clinical trial conducted by our lab, MELVAC, showed that DCs loaded with α-GalCer and peptides encoding immunogenic epitopes for NY-ESO-1 resulted in the expansion of antigen-specific T cells in 7 out of 8 patients[184]. However, the anti-tumour effects of NKT cell-based cancer vaccine strategies are still currently being investigated.

One concern with using NKT cells as cellular adjuvants is the discrepancy in frequency in humans where they represent a substantially smaller population within circulating T cells (> 0.1%) compared to mice (~ 1%)[22,177]. It is unknown yet whether this significant difference in cell number will affect the outcomes of NKT cell-based immunotherapies and therefore require further investigation within the human setting. In contrast, MAIT

cells are highly abundant in humans, representing up to 10% of circulating T cells and are highly enriched across mucosal organs as well as the liver and secondary lymphoid organs[23,25,200]. Their high frequency and the emerging evidence that MAIT cells can influence DC responses suggest that the MAIT activating ligand, 5-OP-RU, may be a useful adjuvant in the design of novel vaccine strategies. Importantly, it is now established that human MAIT cells can activate DCs through CD40L signalling[29], an important mechanism known to boost antigen-specific T cell responses. Thus far, no research has described whether this phenomenon can occur in an *in vivo* setting. Therefore, the aims of this chapter are to explore the capacity of using 5-A-RU, the precursor that binds with cellular sources of MG to form 5-OP-RU, to induce *in vivo* activation of DCs and whether they can adjuvant adaptive T cell responses to co-administered antigens.

Our laboratory has shown that chemical conjugation of peptide antigens via a cathepsin-cleavable prodrug version of α-GalCer leads to improved T cell activity as a result of enhanced co-delivery of the NKT cell agonist and peptide to the same APC[242,243]. Once separated by enzymatic cleavage, both α-GalCer and peptide are simultaneously released and presented on CD1d and MHC-I, respectively. This co-localisation of agonist and peptide results in the licensing of the APC with increased expression of co-stimulatory markers which enhances the engagement of antigen-loaded MHC-I to cognate T cells. *In vivo* administration of such α-GalCer conjugates results in decreased NKT activation, and an overall reduction in circulating IFNγ, relative to injection of unconjugated α-GalCer, but significantly enhances T cell function[242,243]. This was interpreted to suggest that conjugation encourages focused NKT cell-mediated adjuvant activity on relevant APCs involved in T cell immunity. As CD1d can be found expressed on a range of hematopoietic cell lineages and other cell types such as intestinal epithelial cells and liver hepatocytes[256–258], unconjugated α-GalCer alone may be presented by an array of cells to induce the activation of NKT cells that do not possess the molecular machinery to process and present antigenic peptides to T cells efficiently. The fact that cathepsins are highly expressed by professional APCs also makes it more likely that α-GalCer conjugates will be specifically released in APCs capable of inducing antigen-specific T cell responses. In support of this, the capacity of the conjugate vaccines to stimulate $CD8^+$ T cells was reduced in mice depleted of cDC1 cells, which have an enhanced ability for cross-presentation[259].

In addition to increasing potency, the advantage of synthetic vaccines is that the choice of the peptide is highly amendable, providing a useful platform to adapt different antigens easily, and hence target various diseases. The previous chapter described preparation of the 5-A-RU prodrug 10 (referred to as 5-A-RU^{L} from here on). As had been noted, this linker provides an opportunity to append additional motifs such as peptides to 5-A-RU; therefore, this chapter also describes the application of 5-A-RU conjugated to model antigens in order to explore the possibility of enhancing adaptive responses similarly to α-GalCer.

4.2 Aims

Current research on MAIT cells suggests that they have some capacity to modulate adaptive immune responses. Importantly, it is now established that human MAIT cells can activate DCs through CD40L signalling, an important mechanism known to boost antigen-specific T cell responses. Thus far, no research has described whether this phenomenon can occur in an *in vivo* setting. Moreover, in the previous chapter, the synthesis of a 5-A-RU prodrug has provided a potent MAIT cell agonist that enhances *in vivo* activity and will allow the attachment of peptides to drive co-localisation of adjuvant and antigen within a singular DC. Thus, this chapter aimed to assess the ability of MAIT cells to activate DCs to enhance adaptive T cell responses and whether conjugation of 5-A-RU to peptides will improve this response, and could, therefore, be used as a synthetic vaccine strategy.

Specific aims are:

- Determine the ability of 5-A-RU +/- MG to induce MAIT cell and DC activation in secondary lymphoid tissues
- Assess whether administration of 5-A-RU admixed with the antigen leads to generation of antigen specific $CD4^+$ or $CD8^+$ T cell responses
- Determine the mechanism in which 5-A-RU is able to adjuvant T cell responses
- Establish whether the prodrug of 5-A-RU induces DC activation
- Establish whether conjugating peptide to 5-A-RU prodrug enhances specific T cell responses

4.3 Results

4.3.1 Intravenous injection of 5-A-RU activates murine MAIT cells across multiple tissues

It has previously been reported that when mice are intranasally administered 5-OP-RU, MAIT cells localise within the lung and become highly activated[246]. However, MAIT cell populations can be found at varying frequencies in other tissues of specific relevance to initiating immunity, notably the lymph nodes, spleen and liver[23] and capacity for MAIT agonists to activate cells at these sites *in vivo* has not been explored in any detail. Intravenous administration would be expected to deliver small molecules, like MAIT cell agonists, to sites such as the lung, liver, and spleen via the systemic circulation. This route of administration was therefore chosen to conduct an initial assessment of MAIT cell activation in lung spleen and liver, and importantly, because these organs also contain numerous well-defined APC populations, it was also possible to explore the downstream impact of MAIT cell activity on APC phenotype. Groups of mice were injected intravenously with either 5-A-RU or 5-OP-RU prepared immediately before injection by mixing 5-A-RU and MG in the same tube for 2 min. At 18 h after injection, the lung, liver and spleens were collected and the level of MAIT cell and DC activation was compared to vehicle (PBS)-injected controls.

In all organs tested MAIT cells were identified by flow cytometry using MR1 tetramers loaded with 5-OP-RU and an antibody to TCR β, using a gating strategy that involved the exclusion of dead cells, monocytes ($B220^+$) and myeloid cells ($CD11b^+$) (Appendix B). In mice treated with 5-A-RU alone, upregulation of the early activation marker CD69 was observed specifically on MAIT cells in spleen, liver and lung (Fig 4.1a & b), but this did not reach statistical significance in the lung. However, when 5-A-RU was pre-incubated with MG to form 5-OP-RU prior to injection, significant activation of MAIT cells was seen across all these tissues, with the level of upregulation higher than observed in mice treated with 5-A-RU alone. This improved activity of 5-OP-RU over 5-A-RU had also been observed *in vitro* when examining human MAIT cells in chapter 3, and may suggest that there is low cellular availability of endogenous levels of MG to condense with 5-A-RU to form MR1 ligands.

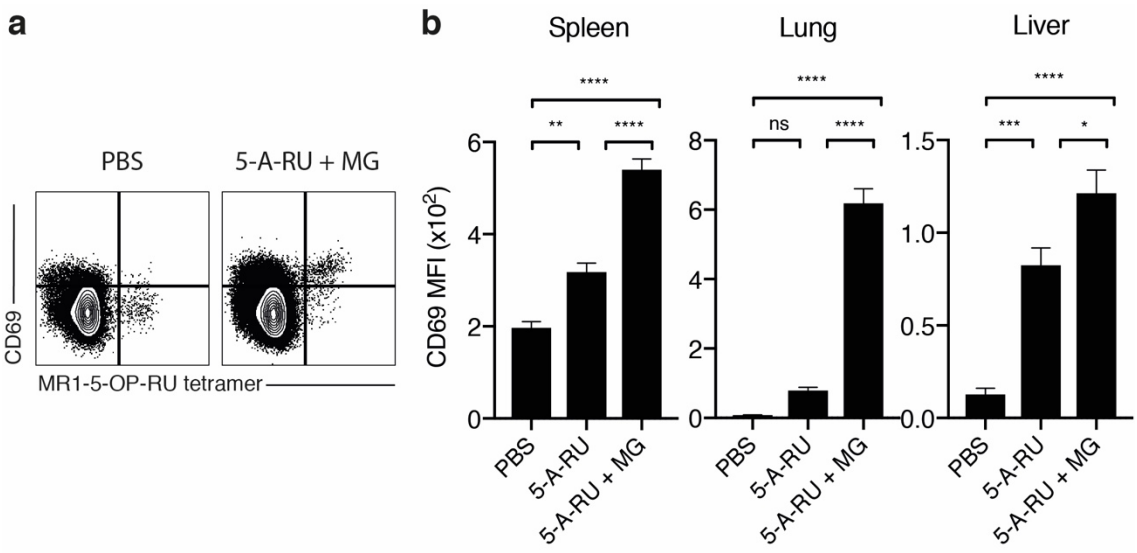


Figure 4.1. Intravenous injection of 5-A-RU leads to activation of murine MAIT cells. Mice (n=5) were administered PBS, 5-A-RU or 5-A-RU + MG i.v. After 18 h, lungs, livers and spleens were harvested and assessed for MAIT cell activation. (a) Representative flow cytometry plots depicting gating strategy for MAIT within the lung and upregulation of CD69 after treatment with 5-A-RU + MG. MAIT cells were identified as MR1-5-OP-RU tetramer^{+} from CD64^{-} B220^{-} TCRβ^{+} cells (b) Graphs depicting the mean fluorescence index (MFI) of CD69 gated on MAIT cells across indicated tissues. Graphs are depicted with the mean ± SEM. Statistical significance was determined by One-Way ANOVA with Tukey's multiple comparisons test. Representative of three similar experiments.

4.3.2 Intravenous injection of 5-A-RU leads to activation of DC subsets and is dependent on T cell signalling.

Previous *in vitro* research on human MAIT cells has shown that upon activation with synthetic agonists, they are able to feedback onto DCs and induce maturation in an MR1- and CD40L-dependent manner[29]. Having established that intravenously delivered 5-A-RU can reach secondary lymphoid organs and robustly activate MAIT cells in mice, it was therefore investigated whether MAIT cells can induce maturation of DCs *in vivo*. Thus, the activation status of DCs from the lung, liver and spleen was examined by flow cytometry 18 h following i.v. injection with 5-A-RU +/- MG. The DCs were divided into two subsets based on their expression of the X-C Motif Chemokine Receptor 1 (XCR1) (Appendix C). The cDC-1 subset, $XCR1^+$, represents a population of *Batf3*$^+$ DCs, that is recognised as an essential subset required in cross-presentation of exogenous antigens onto MHC-I[65]. Conversely, the cDC-2 subset ($XCR1^-$), consist mainly of *Irf4*$^+$DCs are primarily associated with high efficiency of MHC-II antigen presentation[74,75]. For both cDC-1 and cDC-2 subsets, in all tissues, administration of 5-A-RU leads to an upregulation of the T cell co-stimulatory marker, CD86 (Fig 4.2). Interestingly, the use of MG was dispensable despite 5-A-RU + MG inducing a much higher level of MAIT cell activation within each of the tissues tested at the same time point.

To effectively stimulate APCs, T cells must provide activatory signals such as ligation of CD40L with CD40 on the DC[15–17]. Cytokines such as IFN-γ and TNF have also been shown to enhance the magnitude of DC activation[113]. To interrogate whether the activation of DCs by administration of 5-A-RU was reliant on the presence of T cell signalling and pro-inflammatory cytokines, mice were pre-treated with blocking antibodies for CD40L, IFN-γ or TNF via i.p injection 24 h prior and the day of i.v. with 5-A-RU. As shown in Figure 4.3, splenic DC activation in mice treated with 5-A-RU was reduced by the treatment of each of the blocking antibodies but not by their isotype controls.

Together this data demonstrates that administration of 5-A-RU *in vivo* can lead to the maturation of DCs, which is in line with *in vitro* data using human cells. This was observed in organs accessed by intravenous administration that are known to have reasonable quantities of MAIT cells and is dependent on CD40 signalling and provision of cytokines, presumed to be from the activated MAIT cells themselves. This data would

indicate that 5-A-RU could potentially be used as an adjuvant in conjunction with exogenous antigens to produce adaptive immune responses.

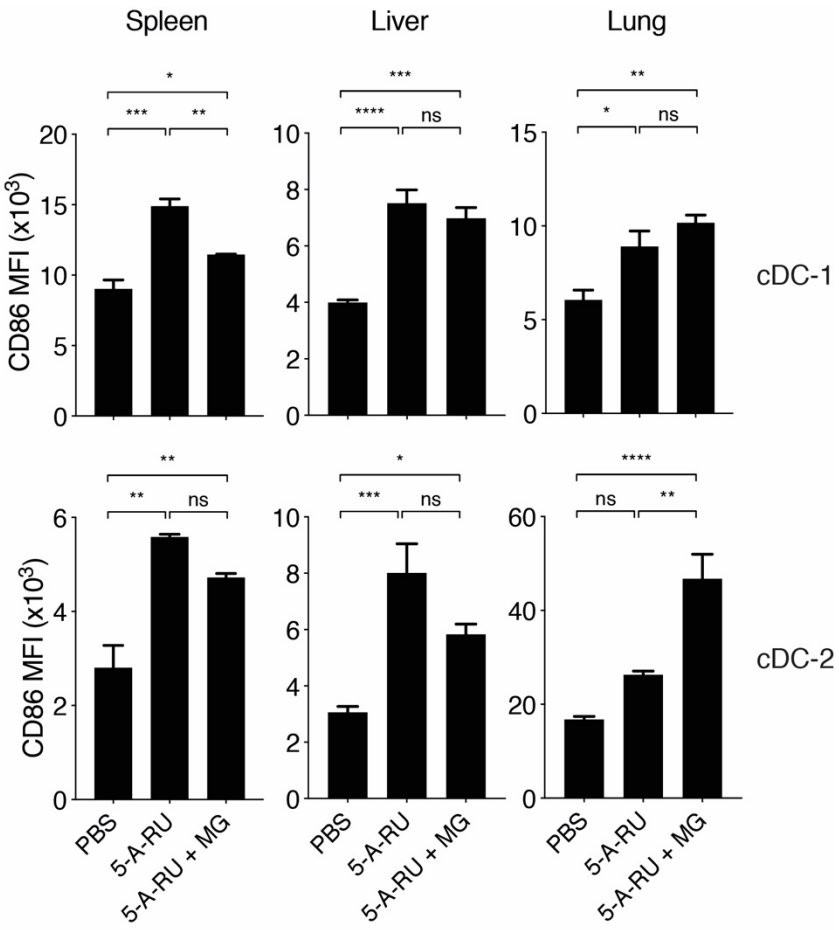


Figure 4.2. Intravenous administration of 5-A-RU induces activation of DCs in lung spleen and liver. Mice (n=5) were administered i.v. PBS, 5-A-RU or 5-A-RU + MG. After 18h, maturation was determined on DCs which were identified as $CD11c^{hi}$ $MHC\text{-}II^{hi}$ cells from $CD64^{-}$ $B220^{-}$ cells. Graphs depict MFI of CD86 expression in each DC subset from treated mice. Data presented is representative of two independent experiments. Graphs are depicted with the mean ± SEM. Statistical significance was determined by One-Way ANOVA with Tukey's multiple comparisons test. Representative of two independent experiments.

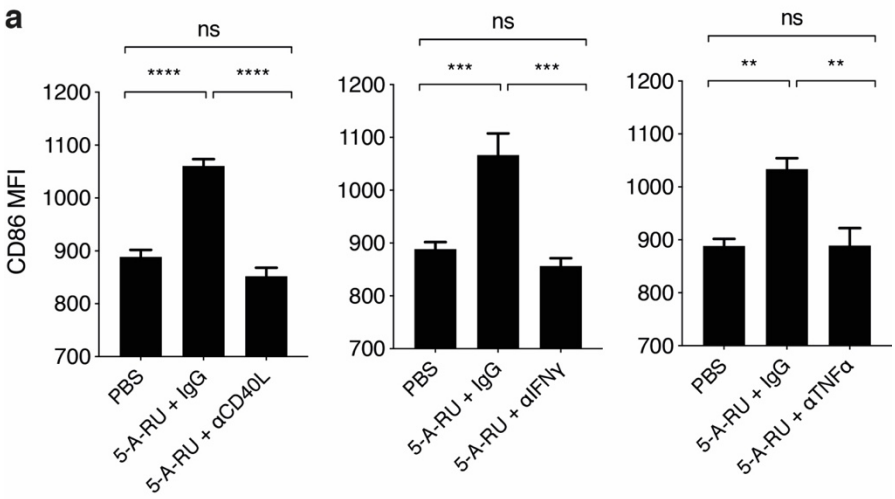


Figure 4.3. 5-A-RU dependent maturation of DCs is mediated by CD40L and pro-inflammatory cytokines. Mice (n=5) were administered i.p. PBS or 500 μg of α-CD40L, α-IFNγ or α-TNFα or their corresponding isotype control armenian hamster IgG, rat IgG1 or human IgG, respectively, on d -1 and d 0. Mice were then administered i.v. PBS or 5-A-RU on d 0 and 18 h later spleens were harvested and assessed for DC activation by flow cytometry. DCs were identified as $CD11c^{hi}$ cells from $CD64^{-}$ $B220^{-}$ cells. Graphs depict MFI of CD86 expression in DC subset from treated mice. The same cohort of PBS treated mice were used as the control group between data sets. Data presented is representative of two independent experiments. Graphs are depicted with the mean ± SEM. Statistical significance was determined by One-Way ANOVA with Tukey's multiple comparisons test. Representative of two independent experiments.

4.3.3 Co-administration of 5-A-RU with antigen induces weak systemic $CD8^+$ T cell responses

It is known that DCs that receive $CD4^+$ T cell help mediated by CD40L-signalling are licensed to induce effective downstream $CD8^+$ antigen-specific T cells through enhanced expression of co-stimulatory molecules and production of important cytokines like IL-12p70[15,16]. As 5-A-RU induces CD40L-dependent activation of DCs the next step was to examine its capacity to adjuvant peptide-specific T cell responses.

To investigate the ability for 5-A-RU to enhance stimulation $CD8^+$ T cell responses to antigen, an *in vivo* killing assay was used (VITAL assay[225]). The mice were prophylactically vaccinated with an admix of 5-A-RU and OVA protein and then seven days later, when the T cell response was expected to be strong, they were each intravenously challenged with fluorescent target cells prepared from splenocytes of syngeneic mice. Three different populations of target cells were used, each pulsed with a different dose of the H-$2K^b$-binding epitope, $OVA_{257-264}$ (SIINFEKL), before injection. The populations were labelled with different concentrations of the fluorescent dye carboxyfluorescein succinimidyl ester (CFSE) so that each target cell population could be gated individually by flow cytometry-based on fluorescence intensity. A control target population was also injected that had not been pulsed with peptide and was stained with CellTracker™ Orange (CTO). Mice that generated a cytotoxic $CD8^+$ T cell response to the peptide target were expected to lyse peptide-loaded target cells, and leave the CTO labelled control population unaffected. Therefore, the proportion of specific lysis could be calculated by establishing the ratio of surviving cells in the CFSE-labelled target populations in blood relative to the CTO labelled control population. Mice that had not been vaccinated, but received vehicle (PBS), served as a negative control on this experiment, while mice injected with OVA mixed with the NKT cell agonist α-GalCer, which was expected to cause a strong $CD8^+$ T cell response, served as a positive control. Example flow cytometry plots are depicted in Fig 4.4a.

Using this method, up to 40 % specific lysis of peptide-pulsed target cells was observed in mice vaccinated with 5-A-RU and OVA (Fig 4.4b) showing that 5-A-RU can function as an immune adjuvant to support cross-priming of a $CD8^+$ T cell response. Again, perhaps surprisingly, prior mixing of 5-A-RU with MG to form 5-OP-RU did not improve the response. It was also clear that the overall magnitude of T cell response was

significantly lower than observed in animals administered OVA and α-GalCer, although α-GalCer is generally regarded as a very potent adjuvant in mice.

Given that the cytotoxic response was relatively weak in mice prophylactically vaccinated with 5-A-RU and OVA, the next step was to determine whether the responses induced could have functional relevance in a disease model. To explore this, mice were engrafted with B16-OVA, a derivative of the murine melanoma cell line B16-F10 which has been transduced to overexpress the OVA protein as a model of a tumour-associated antigen. After challenge with $5x10^5$ B16-OVA cells, tumours were allowed to form over 5 d at which point they became palpable. At this point, mice were vaccinated with 5-A-RU + OVA, a vehicle control or OVA alone. An additional control group was vaccinated with an α-GalCer-OVA conjugate (α-$GalCer^{OVA}$), which has previously been shown to induce potent activity against B16-OVA tumours[243]. While this positive control group exhibited marked anti-tumour activity, mice vaccinated with 5-A-RU + OVA failed to limit tumour growth (Fig 4.5a). This result indicated that MAIT cell activation with 5-A-RU failed to support the priming of a sufficiently powerful antigen-specific T cell response that could delay a fast-growing tumour.

The induction of OVA-specific $CD8^+$ T cells can be detected by flow cytometry using fluorescently conjugated SIINFEKL-H-$2K^b$ pentamers. These molecules consist of five covalently linked H-$2K^b$ molecules (a murine MHC-I molecule expressed in C57BL/6J mice) each bound to an eight amino acid peptide, SIINFEKL, a known $CD8^+$ epitope of OVA ($OVA_{257-264}$). Only $CD8^+$ T cells with a TCR arrangement that binds SIINFEKL in the context of H-$2K^b$ will be bound by the fluorescent pentamer. Using this assay, α-$GalCer^{OVA}$ was shown to be a potent producer of SIINFEKL-specific $CD8^+$ T cells, as expected from the anti-tumour activity observed (Fig 4.5b). In contrast, no significant SIINFEKL-specific $CD8^+$ T cells could be detected in mice at 7 d post-vaccination with 5-A-RU + OVA, thus, while cytotoxic activity could be detected by *in vivo* killing assay, which was conducted in the absence of any tumour, in this experiment, there was little evidence that 5-A-RU + OVA could induce a functional anti-tumour $CD8^+$ T cell response.

It could be reasoned that the weak $CD8^+$ T cell response provided by 5-A-RU, as seen in the *in vivo* killing assay, is unable to overcome tumours once already grafted due to the

immunosuppressive nature of tumour microenvironments. It was, therefore, possible that the T cell response generated with 5-A-RU may be sufficient to prevent initial engraftment of tumours at the peak of the immune response, 7 days after vaccination. To test this, mice were vaccinated prophylactically with 5-A-RU and OVA and challenged one week later subcutaneously in the flank with B16-OVA. Again, the positive control group were vaccinated with α-GalCer and OVA, and tumour growth in the treated animals was compared to growth in animals injected with vehicle only. This analysis showed that mice vaccinated with 5-A-RU +/- MG and OVA were not significantly protected from tumour challenge with B16-OVA (Fig 4.5c). The combination of 5-A-RU + OVA, therefore, could not induce a functional anti-tumour $CD8^+$ T cell response, even when the ratio of T cells to tumour cells was high and solid immunosuppressive tumour lesions had yet to be formed.

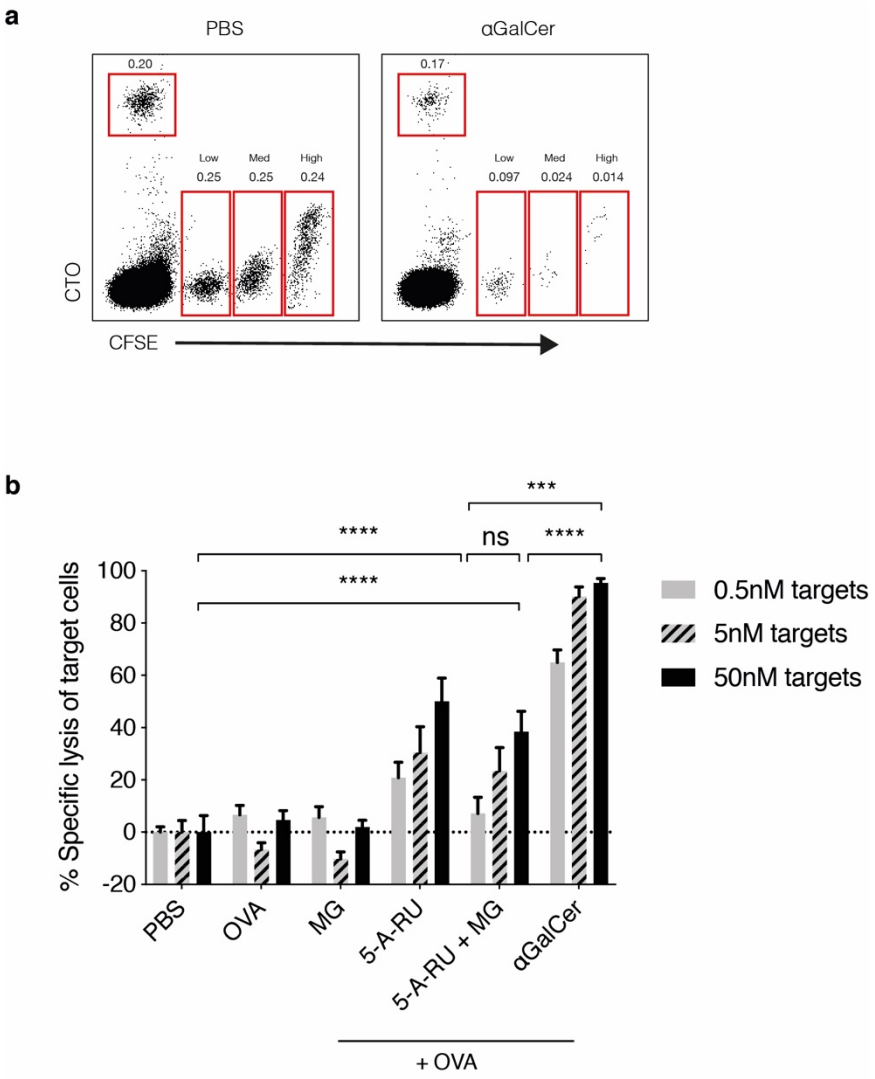


Figure 4.4. Co-administration of 5-A-RU and OVA leads to expansion of OVA specific CD8$^+$ T cells. Mice (n=5) were administered i.v. PBS, OVA or OVA in combination with 5-A-RU, 5-A-RU + MG or α-GalCer. After 7 d, mice were administered CFSE-stained splenocyte populations pulsed with either 0.5, 5 or 50nM of SIINFEKL peptide and equivalent peptide negative control population stained with CTO. Cytolytic activity was measured in the peripheral blood lymphocytes by flow cytometry. (a) Representative flow cytometry plots from treated mice depicting a proportional decrease in peptide-pulsed CFSE-stained target cells in αGalCer + OVA group. (b) Graphs are depicted with the mean ± SEM. Statistical significance was determined by Two-way ANOVA with Tukey's multiple comparisons test. Representative of three independent experiments

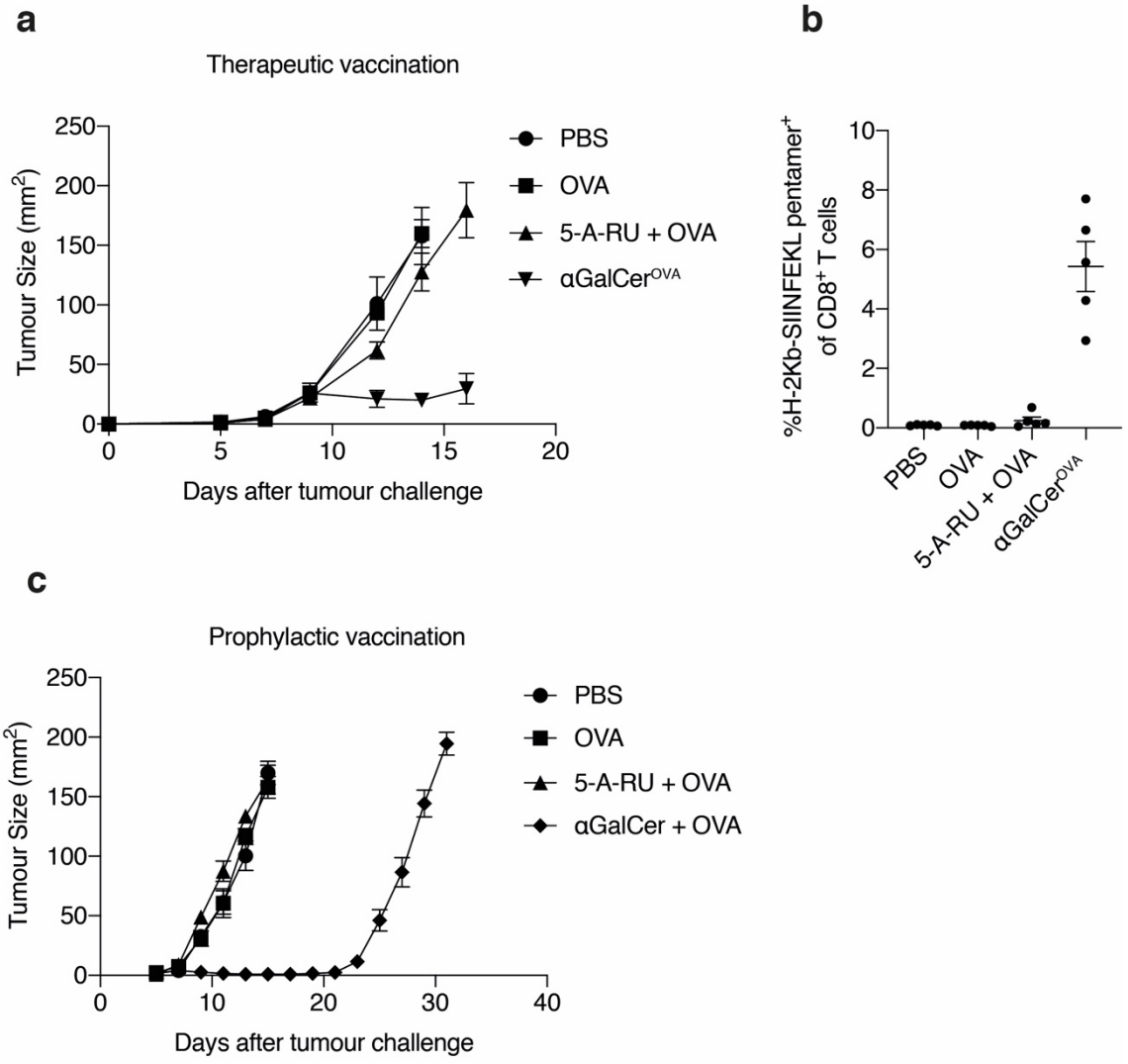


Figure 4.5. Co-administration of 5-A-RU and OVA fail to protect against tumour challenge or expansion of endogenous OVA-specific CD8$^+$ T cells. (a) Mice (n=5) were s.c. challenged with 5x10^5 B16-OVA cells. At 5 d post challenge, mice were administered PBS, OVA, 5-A-RU + OVA or α-GalCer-OVA conjugate i.v. Tumour size was monitored and recorded every 2 d. (b) At 7 d post-vaccination mice were bled and the frequency of H-2K^b-SIINFEKL pentamer$^+$ T cells was assessed by flow cytometry from B220$^-$ CD3$^+$ cells. (c) Mice (n=5) were administered i.v. PBS, OVA or OVA in combination with 5-A-RU or α-GalCer. At 7 d post-vaccination, mice were challenged subcutaneously with 5x10^5 B16-OVA cells. Tumour size was monitored and recorded every two days. Graphs are depicted with the mean ± SEM. Representative of at least two independent experiments

4.3.4 Conjugation of 5-A-RU to OVA peptide enhances OVA-specific CD8+ T cell responses

Previous studies in our lab have demonstrated that targeting peptide antigen and adjuvant to the same APCs by conjugating the components together greatly enhances the generation of peptide-specific T cell responses[242,243]. In the case of α-GalCer-peptide conjugates, after enzymatic processing, these conjugate molecules are able to simultaneously present α-GalCer on CD1d and peptide on MHC-I. These APCs receive signals from iNKT cells to mature the DC through 'helper' signals which in turn enhances the presentation of MHC-I epitopes to naïve $CD8^+$ T cells.

It was, therefore, reasoned that T cell responses generated by co-administration of 5-A-RU and antigen could be enhanced through chemical conjugation of antigenic peptides to the MAIT agonist precursor to facilitate improved uptake and presentation of both components within the same APC. As discussed in the previous chapter, the amino group on 5-A-RU, which condenses with α-dicarbonyl adducts to form MR1-binding ligands, provided a site for attachment of a widely used cathepsin-sensitive VC-PAB carbamate. Incubation with cathepsins or loading onto human PBMCs *in vitro* revealed that this 'pro-5-A-RU' (hereby referred to as 5-A-RU^L) stably released 5-A-RU which in turn could activate MAIT cells.

Thus, a 5-A-RU-linker conjugate was constructed by attaching a bicyclo[6.1.0]non-4-yne (*exo*-BCN) moiety to the valine residue on the original 5-A-RU^L. Any antigenic peptide could then be attached through strain-promoted alkyne-azide cyclo-addition (SPAAC) chemistry (Fig 4.6). To test this vaccine concept, the first peptide conjugated to this structure was KISQAVHAAHAEINEAGRE-SIINFEKLTEWT. All chemical conjugates were synthesised by Ferrier Research Institute This sequence encompassed both a $CD4^+$ T cell epitope (OVA_{323-33}; ISQAVHAAHAEINEAGR) and a $CD8^+$ T cell epitope ($OVA_{257-264}$; SIINFEKL) from OVA protein. Once cleaved, the linker collapses to release 5-A-RU, which in turn condenses with cellular MG to form 5-OP-RU. In order to ensure proper release of the conjugated peptide, the antigenic sequence was preceded by a known protease cleavable sequence (FFRK). Based on our earlier studies with α-GalCer-peptide conjugates, this N-terminal extension is not always required, but in general, enhances processing and presentation regardless of antigenic sequence attached.

In the previous chapter, the protection of 5-A-RU by the VC-PAB linker enhanced its ability to activate MAIT cells. It was hypothesised that this was in part attributed to increased stability of 5-A-RU, allowing enhanced accumulation and specific release within APCs. In line with this hypothesis, this mechanism may also enhance MAIT-DC interactions. Therefore, the 5-A-RU OVA peptide construct (hereby referred to as 5-A-RU^{OVA}) and 5-A-RU^{L} were first tested for their ability to activate DCs compared to the parent molecule 5-A-RU alone. As shown in Fig 4.7a, mice treated with 5-A-RU^{L} or 5-A-RU^{OVA} conjugate both show significantly enhanced DC activation, as shown by enhanced expression of the co-stimulatory molecule CD86, when compared to 5-A-RU alone.

An increase in the ability to activate DCs suggests that 5-A-RU conjugates may also enhance the immunogenic expression of antigen to prime antigen-specific $CD8^{+}$ T cells. To determine the ability of 5-A-RU^{OVA} conjugate to induce these responses, an in vivo killing assay was performed. For these experiments, co-administration of unconjugated KISQAVHAAHAEINEAGRE-SIINFEKLTEWT peptide (hereby denoted as $OVA^{(P)}$) with 5-A-RU or 5-A-RU^{L} was used to assess the need for conjugation. Administration of 5-A-RU or 5-A-RU^{L} admixed with $OVA^{(P)}$ induced a T cell response that achieved around 40% specific lysis of the injected target cells (Fig 4.7b). However, 5-A-RU^{OVA} conjugate induced near 100% lysis of target cells, highlighting the importance of conjugation. Moreover, this response could be titrated to a substantially lower dose before losing activity in killing assay (Fig 4.7c). As 5-A-RU^{L} and 5-A-RU^{OVA} showed similar levels of DC activation, these results support the hypothesis that the co-localisation of antigen and adjuvant within the same DC greatly contributes to the subsequent level of $CD8^{+}$ T cell response.

To further interrogate the strength of the T cell response generated with 5-A-RU conjugates, anti-tumour activity was assessed in mice subcutaneously challenged with B16-OVA cells. At d 5, when tumours were palpable, mice were vaccinated with the conjugate, or with unconjugated components, and then tumour growth was measured every 2-3 d. Mice treated 5-A-RU^{OVA} conjugate were the only group that showed a significant delay in tumour growth post-vaccination (Fig 4.8a). To investigate whether the immune response was correlated to the production of CD8+ T cells specific to

SIINFEKL, mice were bled at 7 d post-vaccination and analysed by flow cytometry. Reflective of the tumour delay, mice that received 5-A-RU^{OVA} were the only group to show a significant expansion of SIINFEKL-specific CD8^{+} T cells (Fig 4.8b).

As the 5-A-RU^{OVA} conjugate induces DC activation to a higher degree than 5-A-RU, it was possible that this enhanced adjuvant activity induced an endogenous anti-tumour response that did not necessarily involve OVA. To rule this out, mice bearing B16-OVA tumours were vaccinated with 5-A-RU^{OVA} or an irrelevant peptide conjugate, 5-A-RU^{GP33}. This construct contains the CD8^{+} T cell epitope for another model antigen lymphocytic choriomenigitis virus glycoprotein, GP33 (KAVYNFATM). As shown, only mice treated with the 5-A-RU^{OVA} conjugate generated a significant tumour growth delay to B16-OVA tumours (Fig 4.8c). This was reflective of the response seen in the blood where only 5-A-RU^{OVA} vaccinated mice showed an expansion of SIINFEKL-specific CD8^{+} T cells (Fig 4.8d).

Together these results demonstrate that the chemical conjugation of 5-A-RU to antigenic peptide significantly improves peptide-specific CD8^{+} T cell responses.

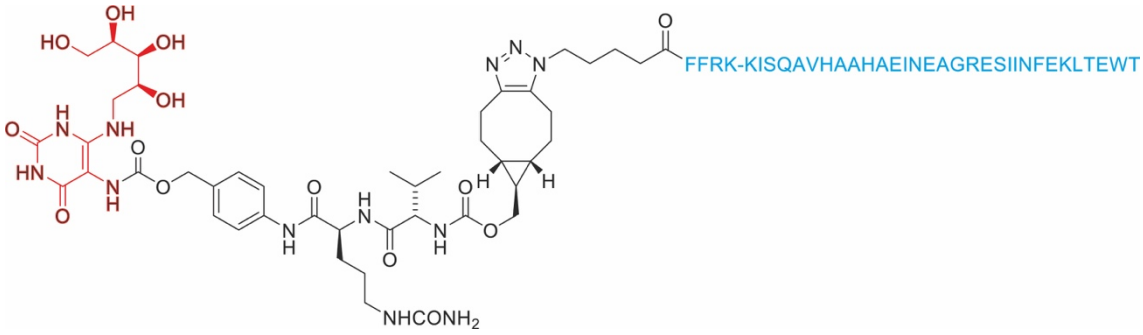


Figure 4.6. Structure of 5-A-RU^{OVA}. Chemical structure depicts 5-A-RU (red) conjugated via PAB-VC-BCN -SPAAC moiety (black) to the OVA$^{(P)}$ peptide sequence (blue).

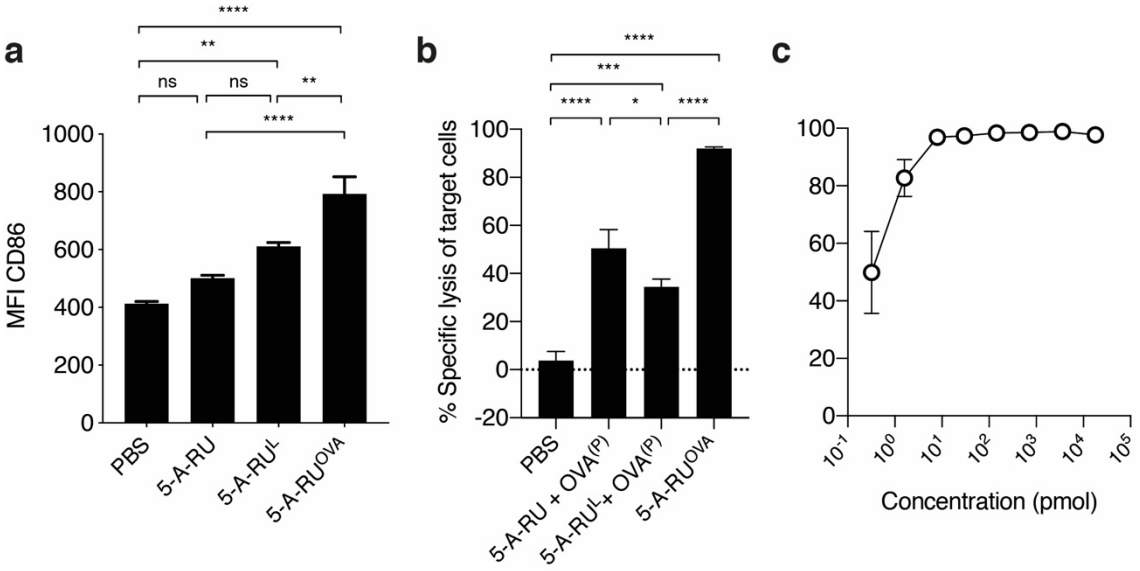


Figure 4.7. Conjugation of 5-A-RU to OVA peptide enhances immune adjuvant activity. (a) Mice (n=5) were administered PBS, 5-A-RU, 5-A-RU-L or 5-A-RU^{OVA} i.v. After 18h, splenic cDCs were assessed by flow cytometry for their expression of CD86. (b) Mice (n=5) were vaccinated with 18nmol of either 5-A-RU^{OVA} or OVA$^{(P)}$ alone or in combination with 18nmol of 5-A-RU or 5-A-RU^{L}. At 7 d post-vaccination, cytolytic activity to peptide-pulsed target cells was measured by the VITAL assay. (c) Titrating amounts of 5-A-RU^{OVA} were administered to mice (n=3) and cytolytic activity to peptide-pulsed target cells was measured by the VITAL assay. Graphs are depicted with the mean ± SEM. Statistical significance was determined by One-Way ANOVA with Tukey's multiple comparisons test. Each data set presented is representative of at least two independent experiments.

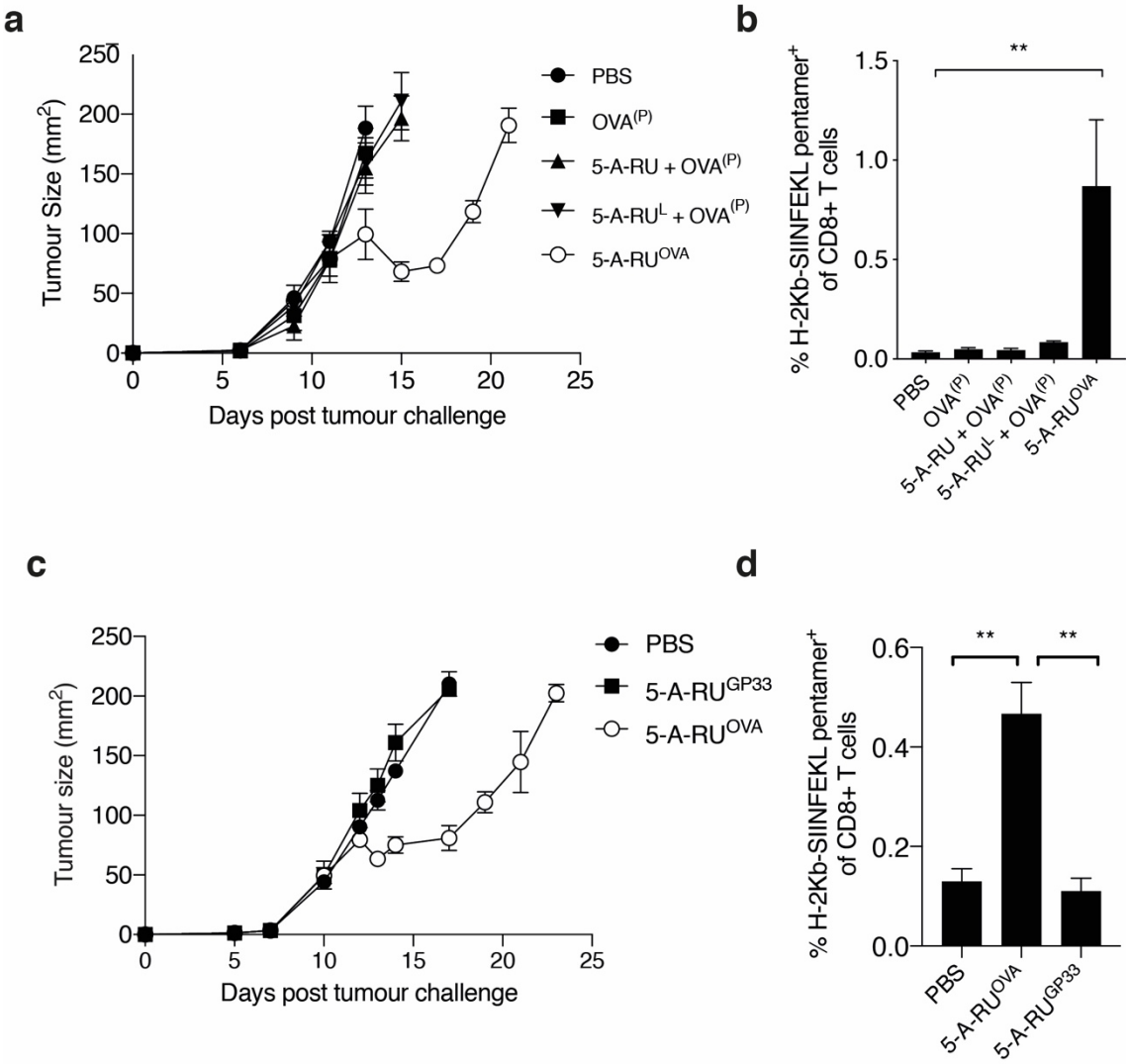


Figure 4.8. 5-A-RU conjugates induce peptide specific protection from tumour challenge. (a) Mice (n=5) were challenged s.c. with $5x10^5$ B16-OVA cells. At 5 d post challenge, mice were administered 5-A-RU^{OVA}, PBS, OVA alone or OVA in combination with 5-A-RU or 5-A-RU^{L} i.v. Tumour growth was monitored and recorded every two days. (b) Seven days post-vaccination mice were bled and the frequency of H-$2K^b$-SIINFEKL $pentamer^+$ T cells was assessed by flow cytometry from $B220^-$ $CD3^+$ cells. (c) Mice (n=3) were challenged s.c. with $5x10^5$ B16-OVA cells. At 5 d post challenge, mice were administered PBS, 5-A-RU^{OVA} or 5-A-RU^{GP33} i.v. Tumour growth was monitored and recorded every 2 d. (d) At 7 d post-vaccination, mice were bled and the frequency of H-$2K^b$-SIINFEKL $pentamer^+$ T cells was assessed by flow cytometry. Graphs are depicted with the mean ± SEM. Statistical significance was determined by One-Way ANOVA with Tukey's multiple comparisons test. Representative of at least two independent experiments.

4.3.5 5-A-RU conjugated to an HPV peptide generates antigen-specific CD8$^+$ T cells and protects from TC-1 tumour challenge

Thus far, OVA has been used as a model antigen to assess anti-tumour responses to vaccination with 5-A-RU conjugates. However, as this protein is not naturally expressed in tumours, 5-A-RU should be combined with a more clinically relevant antigen to evaluate whether this vaccine approach could be translated into humans. To test the general utility of the 5-A-RU conjugate platform, T cell responses to another antigen were assessed. Human papilloma virus (HPV) is an oncolytic virus that is a significant cause of cervical and head and neck cancer. Oncogenesis by this virus is mainly mediated through inappropriate expression of E6 and E7 within basal epithelial cells. To model this cancer in mouse studies, the TC-1 cell line was developed from a lung epithelial cancer cell line, genetically engineered to express E6 and E7 oncoproteins of HPV[260].

To investigate the ability of 5-A-RU to adjuvant immune responses to HPV-related oncoprotein, 5-A-RU^L was conjugated to an H-2D^b binding CD8$^+$ T cell epitope from the HPV E7 protein: RAHYNIVTF (HPV$^{(P)}$). Using an in vivo killing assay as previously described, mice treated with the 5-A-RU HPV conjugate (5-A-RU^{HPV}) induced T cell responses capable of significant lysis of target cells compared to admixed 5-A-RU and peptide (Fig 4.9a). Furthermore, 5-A-RU^{HPV} conferred protection to TC-1 tumour challenge when administered prophylactically (Fig 4.9b). Again using 5-A-RU^{GP33} as an irrelevant peptide control confirmed that the anti-tumour efficacy was antigen-dependent. Finally, 7 d post-vaccination, flow cytometric analysis using RAHYNIVTF-H-2D^b pentamers revealed that mice vaccinated with 5-A-RU^{HPV} conjugate showed significant expansion of HPV E7-specific CD8$^+$ T cells (Fig 4.9c).

Collectively, these results show that 5-A-RU is also capable of adjuvanting alternative peptides that hold more clinical relevance. The data presented is the first to suggest that 5-A-RU is a valid candidate in the use of developing novel tumour immunotherapies.

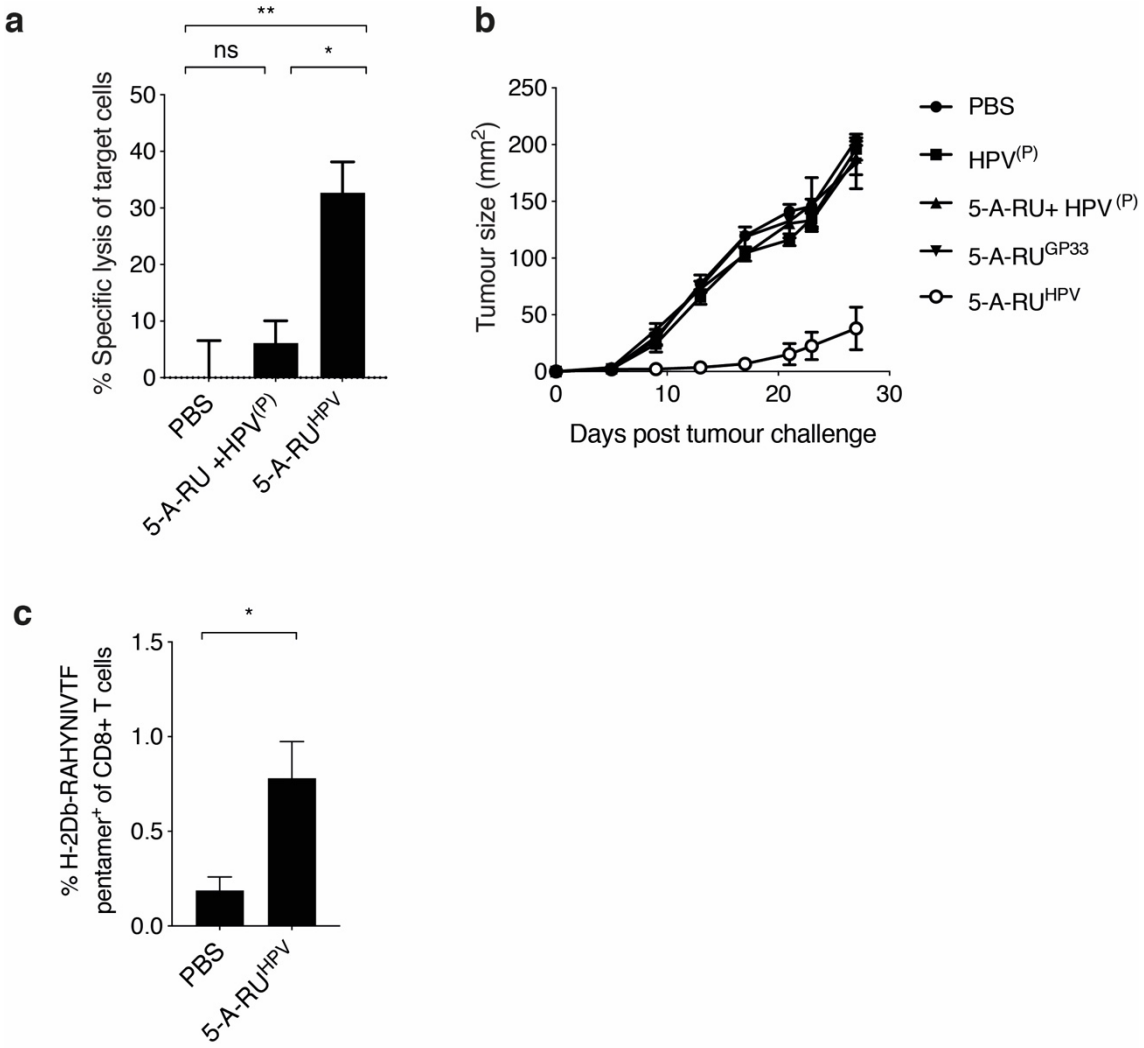


Figure 4.9. Protection from TC-1 tumour challenge with a 5-A-RU HPV conjugate. (a) Mice (n=5) were administered i.v. PBS, 5-A-RU + HPV$^{(P)}$ or 5-A-RU^{HPV}. At 7 d post-vaccination, cytolytic activity to peptide-pulsed target cells was measured by the VITAL assay. (b) Mice (n=5) were administered PBS, HPV$^{(P)}$ +/- 5-A-RU, 5-A-RU^{GP33} or 5-A-RU^{HPV} i.v. At 7 d post-vaccination, mice were challenged s.c. with 1x10^5 TC-1 cells and tumour size was measured every 2 – 3 d. (c) Mice (n=5) were administered i.v. PBS or 5-A-RU^{HPV} At 7 d post-vaccination, mice were bled and the frequency of H-2D^b-RAHYNIVTF pentamer$^+$ T cells was assessed by flow cytometry. Graphs are depicted with the mean ± SEM. Statistical significance was determined by One-Way ANOVA with Tukey's multiple comparisons test. Representative of at least two independent experiments.

4.3.6 The immune response to 5-A-RU^{OVA} is dependent on Clec9A+ cross-presenting DCs

The earlier results in this chapter demonstrated that 5-A-RU and 5-A-RU conjugates could effectively activate cDCs. With subsequent studies showing that 5-A-RU^{OVA} conjugate vaccines also confers protection against tumours, the assumption had been that the mechanism of action was through priming of T cells by MAIT cell-licensed DCs. However, because the expression of MR1 is widespread, it was possible that other cell types may be contributing to the presentation of 5-A-RU derivatives and antigen during priming of the T cell response. Therefore, it was necessary to conclusively define the role of DCs in this vaccine response.

To explore the necessity of the DC compartment, a conditional ablation model was used to deplete DCs *in vivo* based on the expression of the DC-associated marker CD11c. To do this, CD11c-DTR mice were used. These mice express a transgene containing the human diphtheria toxin receptor (DTR) sequence under the control of the CD11c promoter (*Itgax*)[261]. As mice are generally tolerant to low doses of diphtheria toxin, the more sensitive human DTR renders cells expressing CD11c susceptible to the cytotoxic effects of injected toxin, and they will be ablated. However, due to the limited expression of CD11c on more than just immune cells, multiple rounds of DT administration have been shown to be toxic to mice[262]. Therefore, bone marrow chimeras were established in which bone marrow was transferred from CD11c-DTR mice into irradiated C57Bl/6J (Wild-type; WT) mice to ensure the selective targeting of DT to only myeloid cells expressing CD11c. As controls, WT mice were reconstituted with WT bone marrow to assess any immunological effects of transferring bone marrow. After reconstitution with bone marrow from either WT or CD11c-DTR mice, the chimeras were rested for 6 weeks and then were dosed with either DT or PBS 2 d and 1 d before vaccination with 5-A-RU^{OVA} conjugate. Treatment with DT showed specific ablation of cells harbouring CD11c (Fig 4.10a). Seven days post-vaccination, mice who had their DCs depleted showed complete ablation of the CD8$^+$ T cell response as measured by *in vivo* killing assay (Fig 4.10b). These results show that indeed CD11c$^+$ cells, presumably DCs, are the major contributor in the generation of a CD8$^+$ T cell response to 5-A-RU^{OVA}.

Cross-presentation of exogenous antigens onto MHC-I molecules is an important mechanism by which the immune system generates CD8$^+$ T cell responses to many

infections.While $CD11c^+$ cells have the capacity to cross-present antigen to some extent, not all do so to an equal degree. Additionally, not all cells expressing CD11c are DCs. CD11c may also be expressed on a number of other myeloid cells such as macrophages and inflammatory monocytes, and therefore the previous experiment cannot completely inform the critical cell type involved in response to 5-A-RU^{OVA}.

The C-Type Lectin Domain Family 9A (Clec9A) mediates antigen uptake in DCs and is specifically co-expressed on the $CD8^+$ $XCR1^+$ subset[65,263]. Importantly, these DCs, which are commonly referred to as cDC1[67,224], represent the major class of DCs with a heightened propensity for cross-presentation. Given that the 5-A-RU^{OVA} conjugate contains a long peptide which is provided to cells exogenously through vaccination, it was hypothesised that this conjugate would require cross-presentation involving the cDC1 subset. To examine this, Clec9A-DTR mice that allow for depletion of the Clec9A expressing cells with administration of DT were used. The Clec9A-DTR mice were administered i.p. PBS or DT 2 d and 1 d before vaccination with 5-A-RU^{OVA} conjugate and induction of T cell responses were assessed by *in vivo* killing assay. Mice treated with DT showed selective depletion of $CD8^+$ $CD11c^+$ cells, a general phenotype of cDC1 cells in the spleen (Fig 4.10c). Moreover, mice lacking $Clec9A^+$ cells completely lost the ability to generate peptide-specific responses when administered 5-A-RU^{OVA} conjugate (Fig 4.10d). Collectively, these experiments confirm the role of cDC1s and highlight the necessity of cross-presentation in response to 5-A-RU^{OVA}.

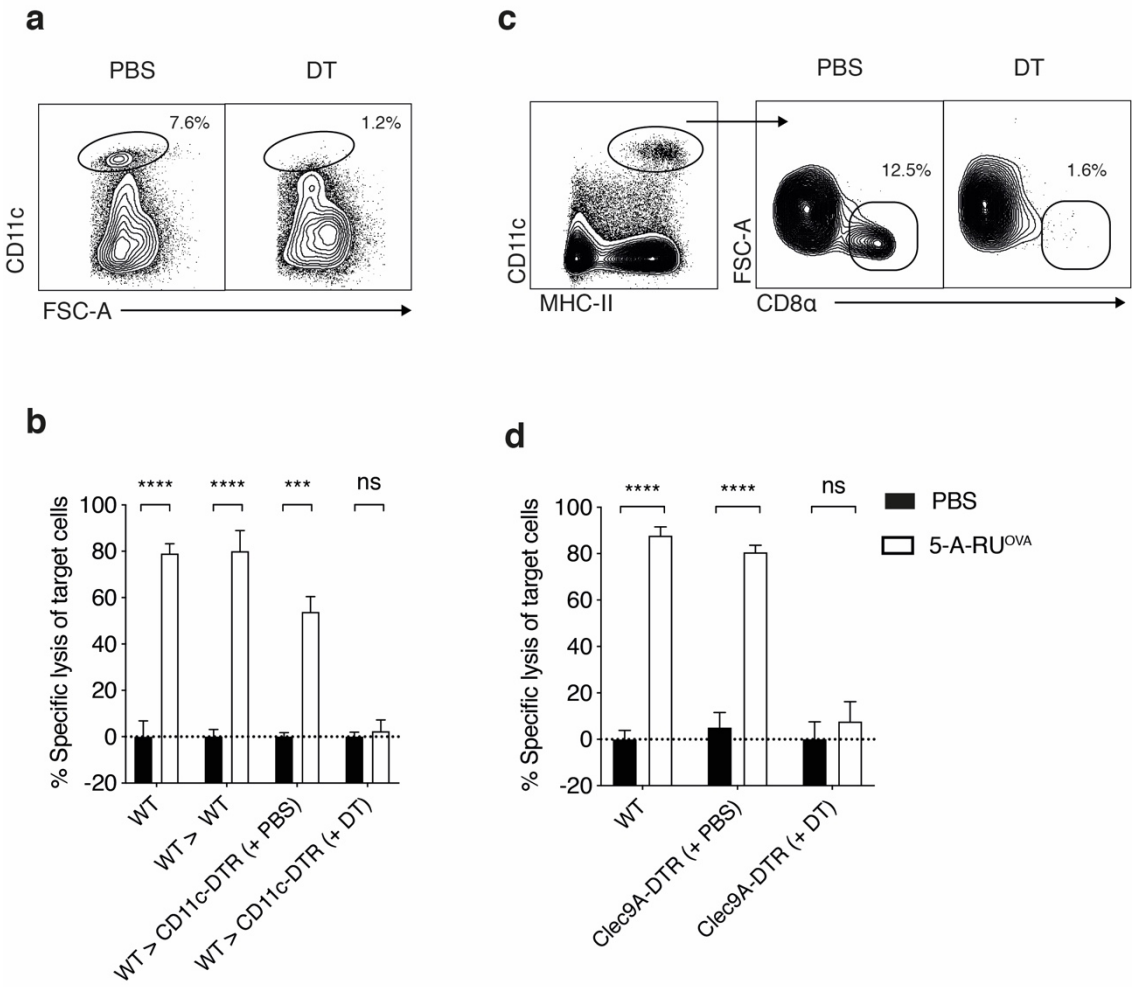


Figure 4.10 Immune response mediated by 5-A-RU^{OVA} is dependent on cross-presenting DCs. Mice (n=5) were irradiated and 24 h later injected with donor bone marrow from either WT mice or CD11c-DTR mice. Six weeks post-reconstitution some mice were treated i.p. with PBS or DT at days -2 and -1 before they were administered i.v. with PBS or 5-A-RU^{OVA} on d 0. (a) Flow plots depicting depletion of CD11c^{hi} cells after DT regime. CD11c^{hi} cells were gated from B220^{-} cells. (b) At 7 d post-vaccination, cytolytic activity to peptide-pulsed target cells was measured by the VITAL assay. (c) WT or Clec9A-DTR mice (n=5) were administered PBS or DT i.p at days -2 and -1 and were administered i.v. PBS or 5-A-RU^{OVA} on d 0. Flow plot depicts depletion of CD8α^{+} DCs after DT regime. (d) At 7 d post-vaccination, cytolytic activity to peptide-pulsed target cells was measured by the VITAL assay. Graphs are depicted with the mean ± SEM. Statistical significance was determined by Two-way ANOVA with Tukey's multiple comparisons test. Representative of at least two independent experiments.

4.3.7 The immune response to the 5-A-RU conjugate remains intact in MR1$^{-/-}$ mice but absent in CD1$^{-/-}$ mice

Thus far, 5-A-RU has proven to be an effective adjuvant that stimulates MAIT cells, activates DCs, and drives immune responses to antigen. Moreover, conjugation of antigen to 5-A-RU significantly increases its ability to facilitate activation of DCs, leading to enhanced CD8$^+$ T cell responses.

However, to confirm the hypothesis that the adaptive immune responses enhanced by 5-A-RU are indeed dependent on MAIT cells, this vaccination strategy must be tested in mice deficient for MR1, which lack MAIT cells. To test the dependence of MR1, WT or MR1$^{-/-}$ mice were given B16-OVA tumours and treated therapeutically at d 5 with either PBS or 5-A-RU^{OVA} conjugate. Surprisingly, there was no significance in the delay of tumour growth between mice treated with 5-A-RU^{OVA} in either strain (Fig 4.11a). This was reflected in the immune response seen in the blood at seven days post-vaccination (Fig 4.11b), where there was no significant difference in the frequency of 5-A-RU^{OVA} conjugate-induced SIINFEKL-pentamer positive CD8$^+$ T cells between WT or MR1$^{-/-}$ mice.

To investigate whether absence of MAIT cells had an impact further upstream of the T cell response, WT, MR1$^{-/-}$ and CD1$^{-/-}$ were treated with PBS or 5-A-RU^{OVA}, and DCs were assessed for activation. In line with the tumour responses, mice lacking MR1 did not show any significant difference in their ability to activate DCs when treated with 5-A-RU^{OVA} (Fig 4.11c). The induction of T cell response to the conjugate was, therefore, dependent on a mechanism that surprisingly did not involve MAIT cells.

Given the role of T cell-mediated signals in the DC activation observed in response to 5-A-RU *in vivo* (Fig 4.2), and that DC activation occurred in such a short timeframe in these experiments (within a day), it was reasoned that another innate-like T cell population might be involved. To investigate this, CD1-deficient mice were used, which lack NKT cells. It is important to note that the murine CD1 locus only contains *CD1d1* and *CD1d2*; therefore, CD1$^{-/-}$ mice lack CD1d-restricted NKT cells. Surprisingly, the observed activation of DCs induced by 5-A-RU^{OVA} and ability to generate antigen-specific T cell responses towards peptide-loaded target cells was abolished in CD1 deficient mice (Fig 4.11c & e). These data together suggest that CD1d-restricted NKT cells may be playing

the key role in the immune response to 5-A-RU-peptide conjugates. To assess this, activation of NKT cells in this model was investigated by flow cytometry gating on CD1d-α-GalCer tetramers (which function similarly to pentameric molecules mentioned above). To assess the activation of NKT cells, expression of the c-type lectin receptor NK1.1, an important functional co-receptor found on NK and NKT cells highly associated with IFNγ production in NKT cells, was evaluated[172,264]. During activation, NKT cells rapidly down regulate this receptor as a mechanism of immune regulation. In mice vaccinated with 5-A-RU^{OVA}, NKT cells showed a marked down-regulation of NK1.1 on gated NKT cells after 18 h; similar downregulation was seen in α-GalCer treated control mice, as a comparator (Fig 4.11d). Thus, administration of 5-A-RU^{OVA} clearly induces activation of NKT cells.

These surprising findings indicate an alternative mode of action of these vaccines that do not involve the canonical MR1-MAIT activation pathway. However, to establish whether the conjugate vaccine construct itself was somehow activating NKT cells, the next step was to examine whether the parent molecule 5-A-RU was acting through the same mechanism. To this end, WT, CD1$^{-/-}$ and MR1$^{-/-}$ mice were administered PBS or 5-A-RU. To further assess the role of NKT cells, an additional mouse strain lacking NKT cells was used. In Jα18$^{-/-}$ mice the gene for TCRα joining region 18 (TRAJ18) is deleted so that the canonical "invariant" TCR expressed by mouse NKT cells (Vα14-Jα18) cannot be formed[265]. Using the two NKT cell-deficient strains (CD1$^{-/-}$ mice and Jα18$^{-/-}$ mice), it could be investigated whether the immune response to 5-A-RU^{OVA} was dependent on NKT cells themselves, or to CD1d presentation of 5-A-RU (or some derivative) to another CD1d-restricted T cell population. As had been seen with the conjugate, in both WT and MR1$^{-/-}$ mice DC activation was observed after the administration of 5-A-RU. (Fig 4.12a). Activation of NKT cells was also observed (Fig 4.12c), as indicated by down regulation of NK1.1 and upregulation of PD-1 and CD69. In contrast, mice lacking CD1, or specifically lacking Vα14-Jα18 NKT cells, were unable to induce DC activation in response to 5-A-RU. This loss in activity was not due to any loss in MAIT cell activation, as in both CD1$^{-/-}$ mice and Jα18$^{-/-}$ mice the MAIT cells all equally upregulated PD-1 or CD69 in response to 5-A-RU (Fig 4.12b).

Collectively, these results show that the immune responses seen in the earlier experiments with 5-A-RU + antigen, or 5-A-RU-peptide conjugates, were unlikely to have involved MR1-restricted MAIT cells, despite showing a marked activation of these cells. These

compounds can, however, induce adjuvant activity that is dependent on CD1d-restricted NKT cells, which become activated after administration.

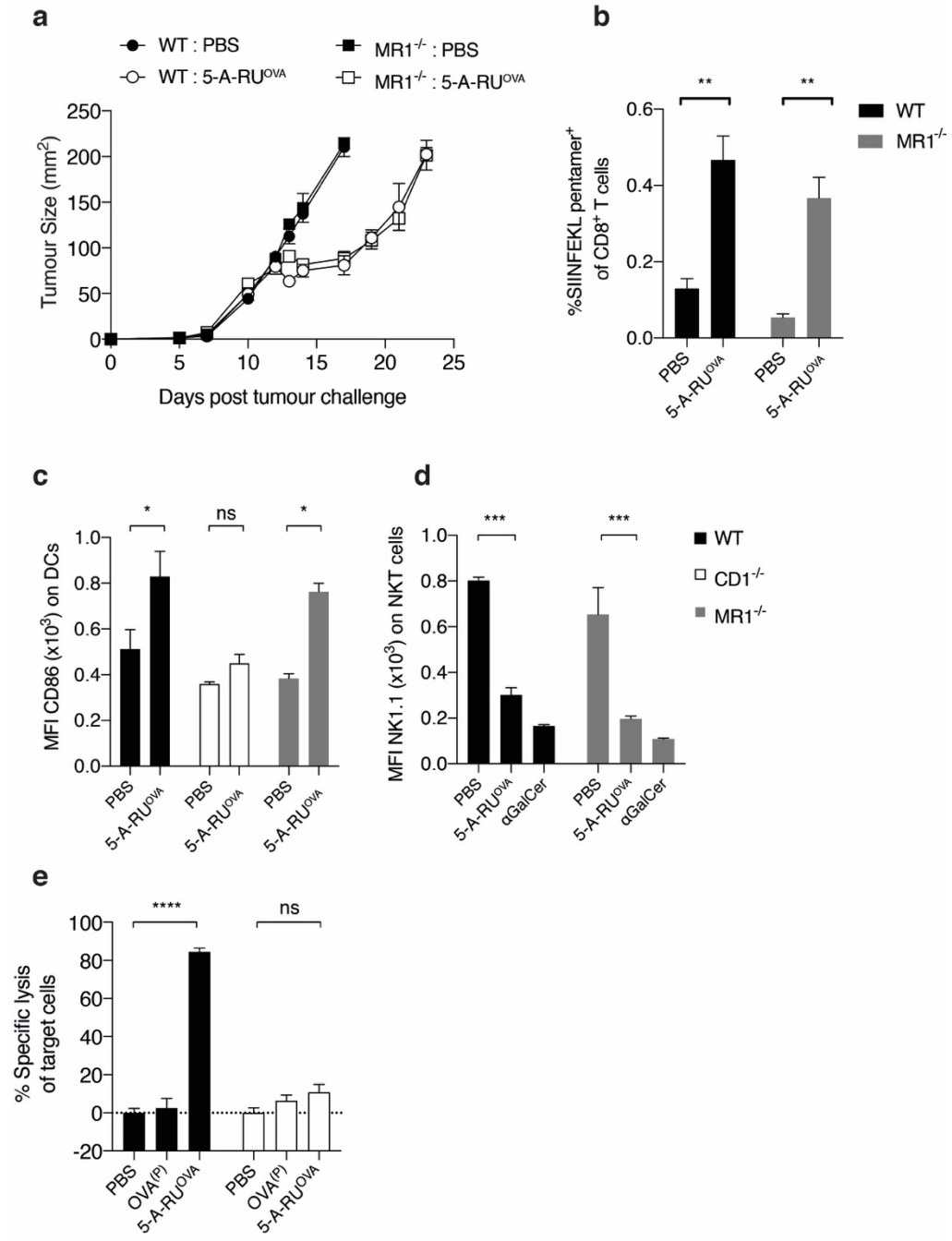


Figure 4.11. Immune response induced by 5-A-RU^{OVA} is dependent on CD1 but not MR1. WT or MR1$^{-/-}$ mice (n=3) were s.c. challenged with 5x10^5 B16-OVA cells. After 5 d, mice were administered i.v. PBS or 5-A-RU^{OVA}. Tumour growth was monitored and measured every 2-3 d. (b) After 7 d, mice were bled and the frequency of H-2K^b-SIINFEKL pentamer $^+$ T cells was assessed by flow cytometry. (c) WT, MR1$^{-/-}$ or CD1$^{-/-}$ mice (n=3) were administered i.v. PBS or 5-A-RU^{OVA}. After 18 h, spleens were harvested and DCs were assessed by flow cytometry from MHC-II^{hi} CD11c^{hi} B220$^-$ CD64$^-$ cells for expression of CD86. (d) Mice (n=3) were administered i.v. PBS, 5-A-RU^{OVA} or α-GalCer. After 18 h, spleens were harvested and NKT cells were assessed by flow cytometry as CD1d-α-GalCer tetramer$^+$ B220$^-$ TCRβ$^+$ cells. MFI of NK1.1 was calculated on NKT cells. (e) Mice (n=5) were administered PBS, OVA$^{(P)}$ or 5-A-RU^{OVA} i.v. At 7 d post-vaccination, cytolytic activity to peptide-pulsed target cells was measured by the VITAL assay. Graphs are depicted with the mean ± SEM. Statistical significance was determined by Two-way ANOVA with Tukey's multiple comparisons test. Representative of at least two independent experiments.

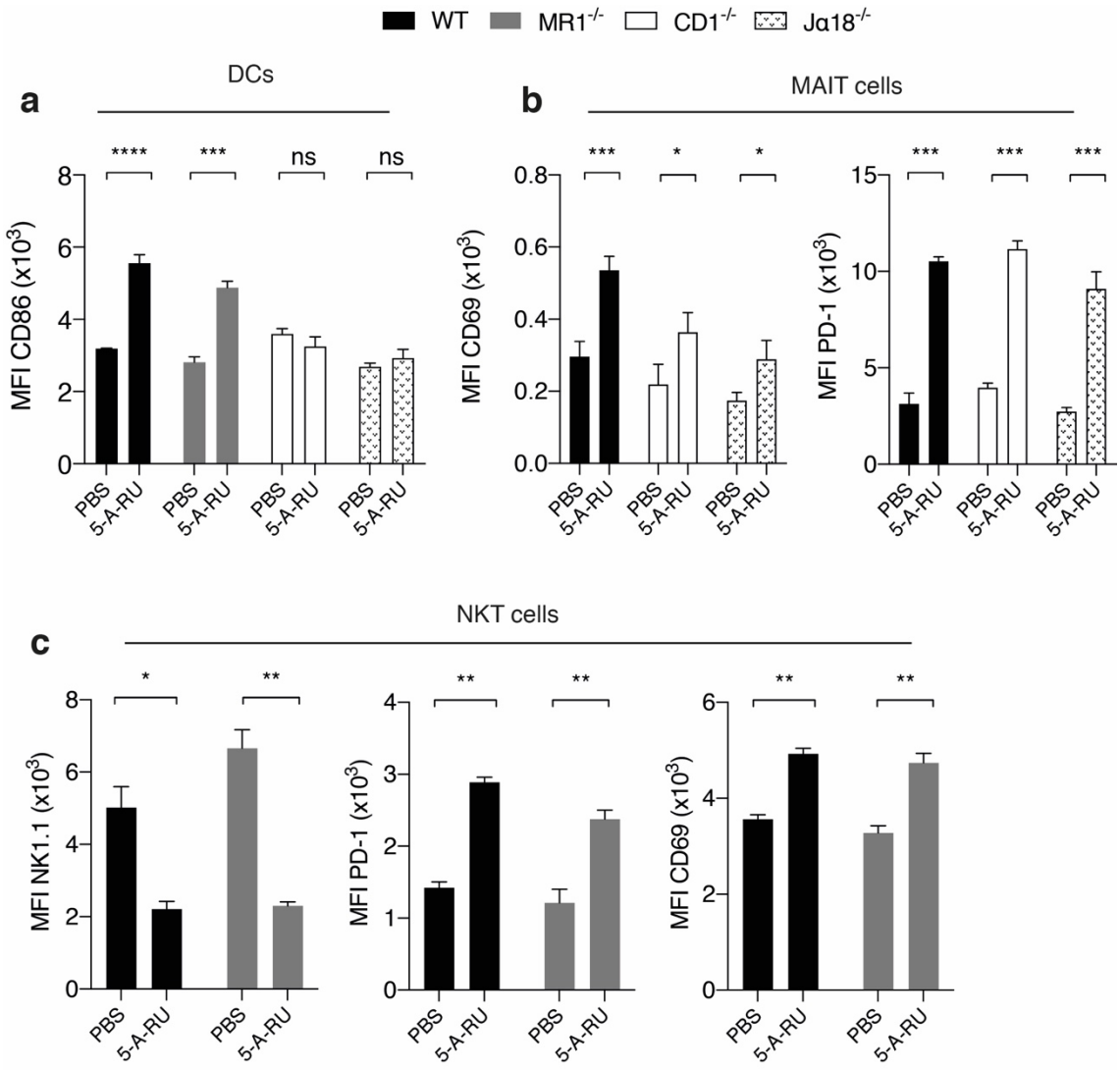


Figure 4.12 5-A-RU mediated DC maturation is dependent on the presence and activation of NKT cells. WT, MR1$^{-/-}$, CD1$^{-/-}$ or Jα18$^{-/-}$ mice (n=5) were administered i.v. with PBS or 5-A-RU. After 18 h, spleens were harvested and assessed by flow cytometry. (a) Expression of CD86 was measured on cDCs. (b) Activation of MAIT cells was assessed by the expression of CD69 or PD-1. (c) Activation of NKT cells was assessed by expression of CD69, PD-1 or NK1.1. Graphs are depicted with the mean ± SEM. Statistical significance was determined by One-way ANOVA with Tukey's multiple comparisons test. Experiments a and b were from one experiment and c was representative of two independent experiments.

4.3.8 Fractionation and filtration of 5-A-RU reveals its NKT and DC activity is dependent on an unknown micro-particle contaminant

The observation that NKT cells were required for DC activation, and hence the adjuvant activity of 5-A-RU or 5-A-RU-conjugates, suggests that the 5-A-RU-based compounds used in the previous experiments may contain some NKT-activating contaminant, or that the 5-A-RU itself forms a CD1d-binding ligand able to activate NKT cells directly. In order to assess the former, Dr Regan Anderson (Ferrier Research Institute) used LCMS in an attempt to identify any α-GalCer that may be contaminating the 5-A-RU preparations. However, no evidence could be found to suggest that this was the case (data not shown). To further interrogate this hypothesis, 5-A-RU was subjected to preparative HPLC, a method employed in chemistry in order to isolate compounds based on their size and physicochemical properties. This method would allow isolation of 5-A-RU and exclusion of any material with alternative molecular masses or physicochemical properties (Fig 4.13). After this process, 5-A-RU could be correctly identified in an early soluble fraction and was isolated for study (Appendix A). In the previous chapter, 5-A-RU was shown to be labile to auto-oxidation. Thus, to establish whether fractionation had affected the integrity of 5-A-RU as an active ligand, this 'fractionated' 5-A-RU was then tested for its ability to activate human MAIT cells. As shown in Fig 4.14a, fractionation of 5-A-RU did not affect its ability to activate human MAIT cells when compared to the same batch which had not undergone fractionation. Interestingly, when this fractionated 5-A-RU was injected intravenously into mice, it was unable to activate splenic DCs or NKT cells (Fig 4.14b and c).

Given that the laboratory that produced 5-A-RU used for these studies has a long-standing history of synthesis of α-GalCer, including analogues or peptide-conjugate forms of α-GalCer, it was speculated that the contaminating product could be derived from particulate matter such as silica from preparative steps to which trace amounts of varying CD1d-binding ligands may have adhered, and which would not be detectable by LCMS. Therefore, 5-A-RU was filtered by centrifugation through a tube with a 0.1μM pore filter in order to investigate whether particulate matter contributes to the responses seen. When tested on human PBMCs, filtered 5-A-RU did not show any deficiency in the ability to activate MAIT cells (Fig 4.15a). However, in a similar fashion to fractionated 5-A-RU, filtered 5-A-RU lost the ability to activate both splenic DCs and NKT cells *in vivo*.

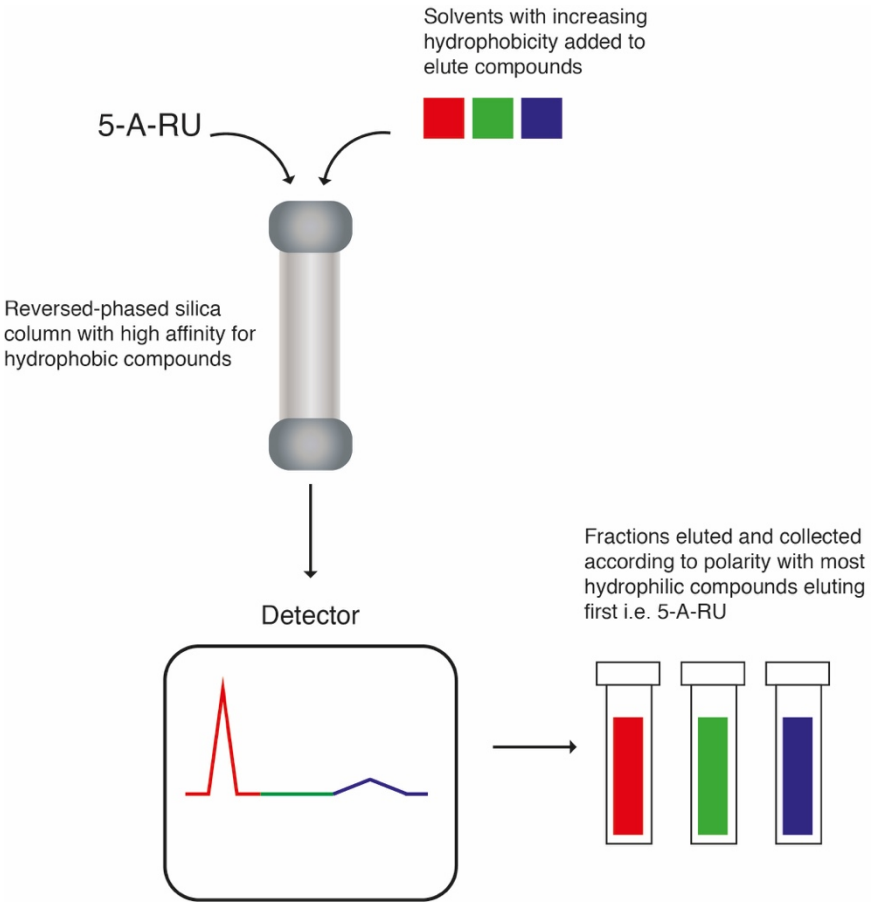


Figure 4.13. Schematic depicting preparative HPLC to fractionate 5-A-RU. Isolation was performed using a Phenomenex Gemini C18 reverse-phased column. A 500µl sample 5-A-RU at 13mgmL^{-1} was loaded onto the column for isolation. Elution was performed by the addition of a mobile phase to the column as follows: 0 – 5 min, 95% water + 5% methanol, 5 – 15 min, 95% water + 5% - 100% methanol (linear gradient), 15 – 20 min, 100% methanol, 20 – 40 min, 90% methanol + 10% isopropanol. All solvents contained 0.05% trifluoroacetic acid. The solvents were applied to the column at a flow rate of 5mL/min. UltraViolet light at 214nm was utilised to identify compounds by spectroscopy. Fraction 1 containing 5-A-RU was detected and eluted between 2 min – 7 min 40 s. Performed by Regan J Anderson

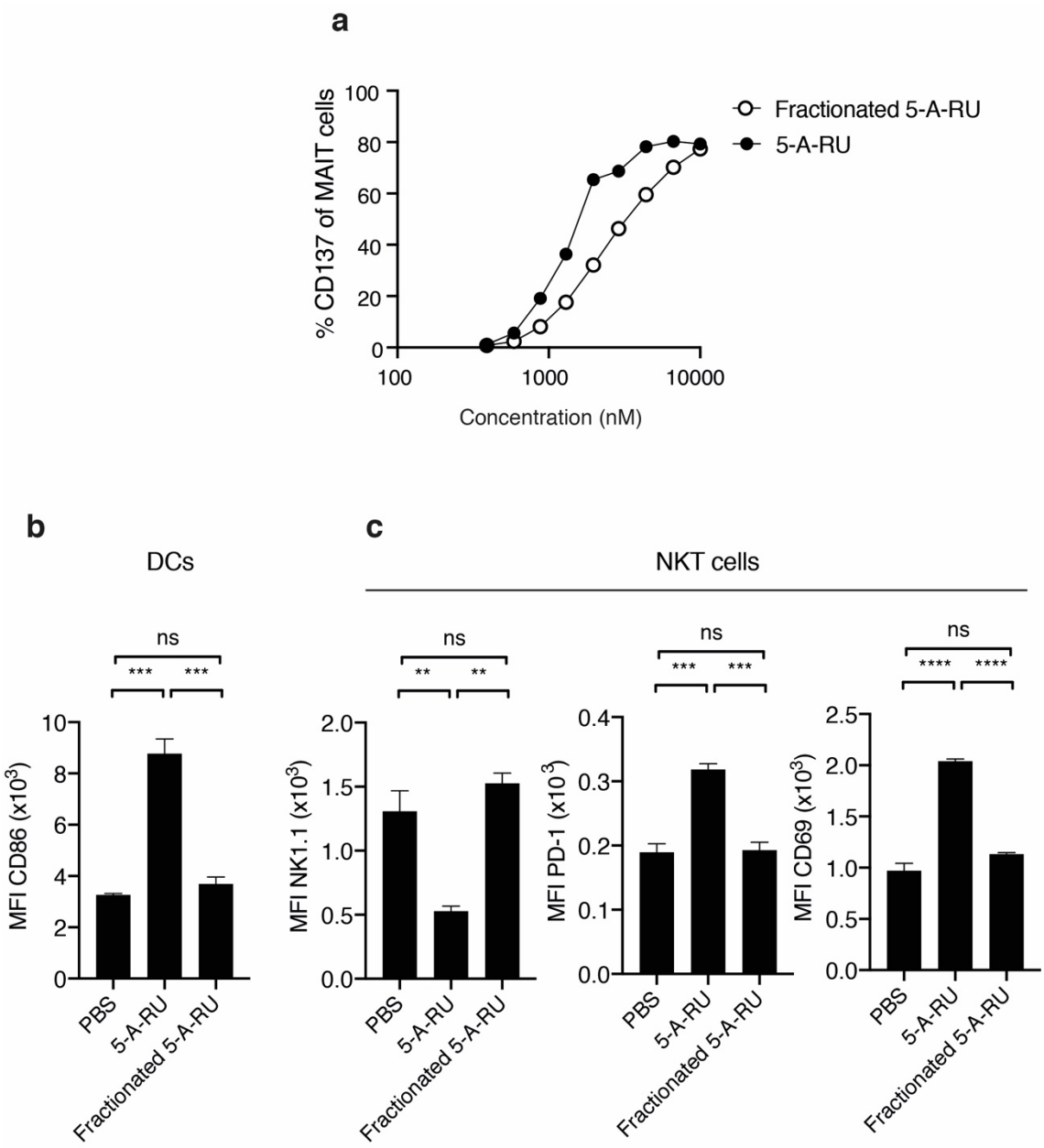


Figure 4.14. Fractionation of 5-A-RU leads to loss in DC and NKT activity. 5-A-RU was fractionated by preparative HPLC. (a) 5-A-RU or 5-A-RU fractionated from the same stock was incubated at titrating concentrations with human PBMCs. After 18h, MAIT cells from the PBMCs were analysed by flow cytometry for activation. MAIT cells were identified as $CD19^{-}$ $CD3^{+}$ $V\alpha7.2^{+}$ $CD161^{hi}$ cells and activation was assessed by expression of CD137 on MAIT cells. Mice (n=5) were vaccinated i.v. with PBS, 5-A-RU or fractionated 5-A-RU. After 18h, spleens were harvested and (b) expression of CD86 was measured on cDCs or (c) expression of NK1.1, PD-1 or CD69 was measured on NKT cells. Graphs are depicted with the mean ± SEM. Statistical significance was determined by One-way ANOVA with Tukey's multiple comparisons test. Data was obtained from a single experiment.

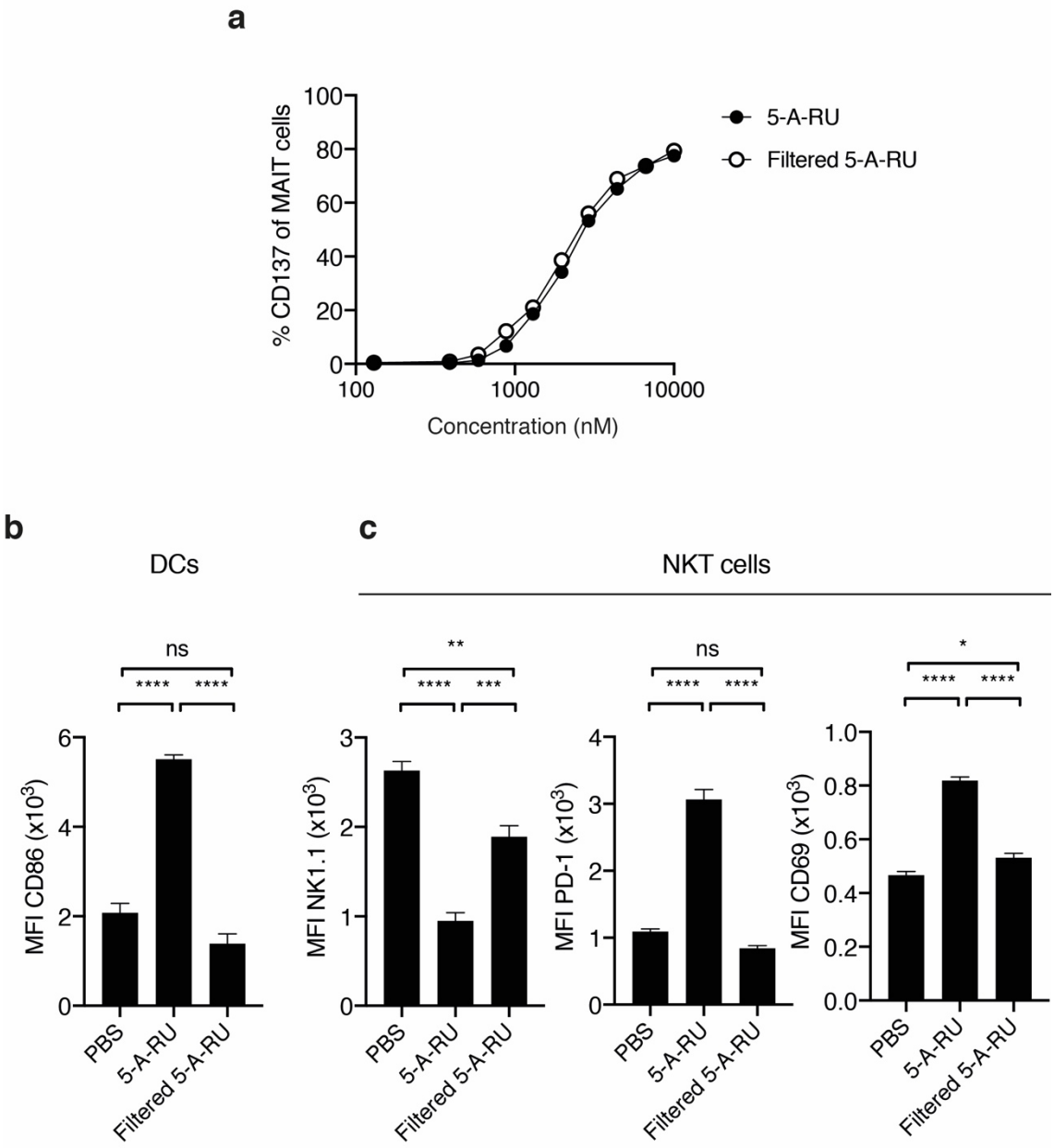


Figure 4.15. Filtration of 5-A-RU results in loss in DC and NKT cell activity. 5-A-RU was filtered through a 0.1µM pore filter by centrifugation (a) 5-A-RU or filtered 5-A-RU from the same stock was incubated at titrating concentrations with human PBMCs. After 18h, MAIT cells from the PBMCs were analysed by flow cytometry for activation by expression of CD137. Mice (n=5) were vaccinated i.v. with PBS, 5-A-RU or fractionated 5-A-RU. After 18h, spleens were harvested and (b) expression of CD86 was measured on cDCs or (c) expression of NK1.1, PD-1 or CD69 was measured on NKT cells. Graphs are depicted with the mean ± SEM. Statistical significance was determined by One-way ANOVA with Tukey's multiple comparisons test. Data was obtained from a single experiment.

4.3.9 CD1d-indendepent maturation of lung DC populations can be achieved when 5-A-RU is admixed with MG

It was possible during the extensive work researching the adjuvant activity of 5-A-RU and 5-A-RU conjugate vaccines that the contaminating NKT cell activity was obscuring any MR1-dependent activity provided by MAIT cells. When 5-A-RU was filtered or fractionated, and therefore devoid of NKT activity, the manipulated material could not induce activation of DCs in the spleen, despite retaining its ability to activate MAIT cells. However, the size of the population of MAIT cells in the spleen is small (< 0.1% of all T cells), which may be insufficient to induce DC activation. As the lung contains one of the highest proportions of MAIT cells in mice, it was therefore decided to re-evaluate the potential for MAIT cells within the lung to activate DCs.

Importantly, in Fig 4.1, it was shown that the addition of MG to 5-A-RU to form 5-OP-RU prior to injection greatly enhanced the activation of MAIT cells, most notably in the lung. Thus, for these experiments, WT or $CD1^{-/-}$ mice were administered purified 5-A-RU with or without prior mixing with MG. In the lungs of 5-A-RU + MG treated mice, there was a significant upregulation of CD86 on both cDC-1 and cDC-2 (Fig 4.16a). Critically, this response was independent of CD1d as $CD1^{-/-}$ mice showed a similar response. While 5-A-RU induced a small increase in CD86 by the DC subsets, this did not reach statistical significance. Interestingly, mice that received 5-A-RU + MG did not show any significant increase in DC activation within the spleen or the liver despite having shown potent MAIT activation in the same tissues (Fig 4.16b). Overall, this data indicates that activation of MAIT cells can promote DC activation within the lungs and that this is independent of NKT cells. However, only when 5-A-RU was first converted to 5-OP-RU by the addition of MG was this apparent, suggesting that availability of sufficient ligand is a limiting step. Further characterisation of this response and analysis in $MR1^{-/-}$ mice will be covered in the following chapter.

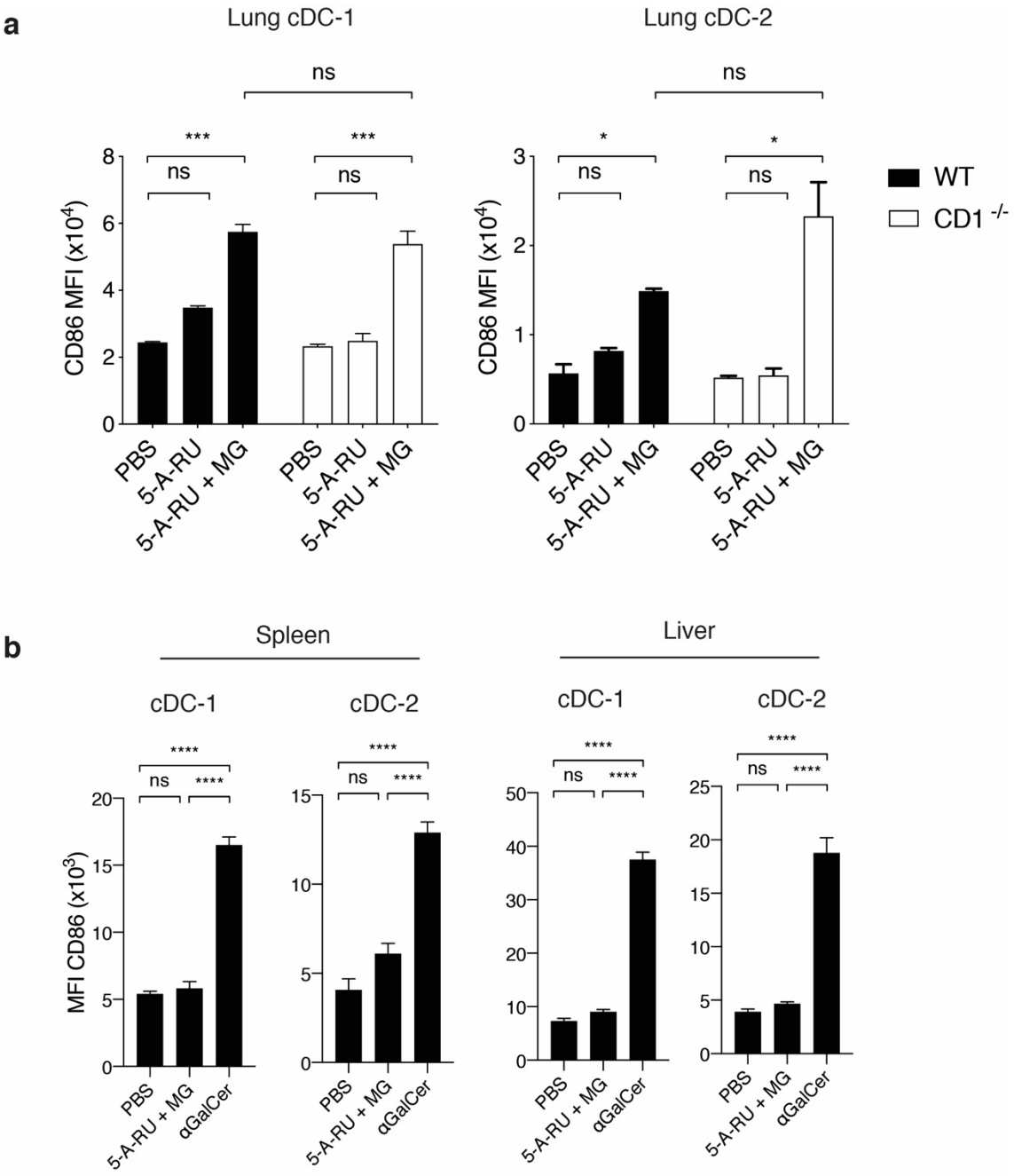


Figure 4.16. 5-A-RU + MG induces CD1d-independent activation of lung DCs. (a) WT or CD1$^{-/-}$ mice (n=5) were administered PBS, purified 5-A-RU or purified 5-A-RU + MG i.v. After 18 h, lungs were harvested and stained for flow cytometry. MFI was calculated for CD86 on lung cDC-1 and cDC-2. (b) Mice (n=5) were administered PBS or 5-A-RU + MG i.v. After 18 h, spleens and livers were harvested and stained for flow cytometry. MFI was calculated for CD86 on lung cDC-1 and cDC-2. Graphs are depicted with the mean ± SEM. Statistical significance was determined by Two-Way ANOVA with Tukey's multiple comparisons test for (a) and a One-Way ANOVA with Tukey's multiple comparisons test for (b). Data presented is from a single experiment.

4.4 Discussion

Previous research conducted on human MAIT cells *in vitro* has revealed that similar to other innate-like T cells[19,20,255,266], they are able to provide stimulation to APCs that present 5-OP-RU on MR1[29]. Upon activation, provision of CD40L by MAIT cells drives the differentiation and activation of immature monocyte-derived DCs to produce IL-12p70 in an MR1-dependent manner[29]. This chapter aimed to assess whether MAIT cells could provide these helper signals towards DCs *in vivo* in order to determine whether MAIT cells could be harnessed as adjuvants for the development of new vaccines and immunotherapies. In the studies presented at the beginning of this chapter, the 5-A-RU material used was shown to be able to activate DCs in a CD40L-dependent manner and that co-administration of 5-A-RU with OVA could indeed adjuvant weak $CD8^+$ T cell responses. Furthermore, the development of 5-A-RU-peptide conjugates not only enhanced DC activation but drove peptide-specific responses that could delay tumour growth. Unfortunately, analysis in $MR1^{-/-}$ mice revealed that the 5-A-RU-mediated responses generated with these materials were not dependent on MAIT cells. In fact, i.v. administration of 5-A-RU and 5-A-RU-peptide conjugate lead to CD1d-dependent activation of NKT cells. However, it was possible to remove the NKT cell-activating component within 5-A-RU by changes to the preparative method. When this was conducted, and 5-A-RU was converted to 5-OP-RU before injection, activation of MAIT cells was observed *in vivo* that lead activation of DCs within the lung where MAIT cells are numerically rich. Interestingly, this DC activation was only observed in the lung but not the spleen or liver where MAIT cells could be seen activated at the same time point. These observations point to the possibility that MAIT cells may indeed be able to adjuvant T cell responses; however, confounding NKT activation within the 5-A-RU compounds obscured any MAIT cell-based activity.

The first hypothesis was the possibility that 5-A-RU, or some derivate thereof, was possibly binding to CD1d in order to activate NKT cells. Although CD1d molecules exhibit some level of promiscuity in what antigens they bind, in contrast to other MHC-I molecules and MR1, the CD1d antigen-binding pocket is exceptionally hydrophobic and lack hydrogen-bonding sites[267,268]. The hydrophobic and large internal A' and F' pockets which typically bind large lipids such as α-GalCer would therefore be unfit to bind a small polar molecule such as 5-A-RU. However, in an attempt to reconcile whether 5-A-RU

was the causal agent which induced NKT and DC activation, preparative HPLC was performed on 5-A-RU in order to isolate 5-A-RU from other possible compounds with alternative physicochemical properties. This was necessary as 5-A-RU was prepared as a crude mix from the reduction of nitrouracil by sodium dithionite which underwent no purification prior to *in vivo* use. This meant that 5-A-RU which would eventually be injected into mice would have trace amounts of non-5-A-RU by-products such as sulfonated derivatives (described in Chapter 3). Analysis of the 5-A-RU fractionated by HPLC revealed little defect in its ability to activate human MAIT cells and was subsequently injected i.v. into mice to assess NKT and DC activation. As shown, compared to 5-A-RU which underwent no purification, fractionated 5-A-RU (without being admixed with MG) completely lost the ability to activate NKT cells and DCs. This result suggests that MAIT cell activation induced by 5-A-RU alone itself was unable to adjuvant T cell responses, and there was some contaminating element causing NKT and DC activation.

Our laboratory's long-standing collaboration with the Ferrier Research Institute has resulted in the synthesis of large amounts of α-GalCer and various lipid analogues over the years. Although stringent cleaning and quality control protocols are in place to prevent cross-contamination between compounds, it was hypothesised that a steady accumulation of trace amounts of lipids could be the source of NKT activity in 5-A-RU preparations. One potential source of these lipids could be from the argon chambers used to store chemical compounds. These chambers are closed systems that fill with argon to displace oxygen in order to prevent oxidation of compounds. It is possible that during the displacement of oxygen, trace amounts of the compounds being stored may become lodged within the gas pumps and chamber; this could be a source of contamination when a subsequent compound is stored, with the flux in air and argon causing the contaminant to be dislodged. Methods to investigate this possibility are currently under investigation. Coincidentally, during the investigation of NKT-based activity generated by 5-A-RU and 5-A-RU conjugates, a new laboratory was built to replace the laboratory in which α-GalCer-related compounds had been generated. A strong piece of evidence that the source of NKT-activity in these 5-A-RU-based compounds is from contamination is the observation that all subsequent 5-A-RU batches produced in the new lab lacked any detectable NKT cell activating properties. However, no such further synthesis of 5-A-RU^{OVA} has been carried out to substantiate these observations further.

It is widely known that α-GalCer is a highly potent activator of NKT cells and is able to activate NKT cells at very low concentrations *in vitro* and *in vivo*. In our experience, i.v. administration of α-GalCer at a dose as low as 2ng (~2.5pmol) per mouse still resulted in NKT-dependent activation of DCs[259]. Surprisingly, despite the observations on activation of NKT cells by 5-A-RU, analysis by LCMS on the original 5-A-RU and 5-A-RU^{OVA} compounds revealed no detectable levels of α-GalCer (data not shown, performed by Regan Anderson). However, other analogues or α-GalCer derivatives were not investigated in this manner, and it is possible that the NKT activity could be accounted for by co-operation of other potential agonists at minute concentrations.

It is important to note that the NKT-activating observations made in these experiments were also seen in a separate collaborator's laboratory who also work on NKT cells (Salio, *personal communication*). In their hands, i.v. administration of 5-A-RU also resulted in the activation of DCs which was abrogated in both β2m$^{-/-}$ (which lack MHC-I restricted T cells) and CD1$^{-/-}$ mice. Importantly, the 5-A-RU used in these experiments was synthesised in another chemistry laboratory. These similar NKT-dependent findings from two separately generated 5-A-RU batches indicates either that contamination with NKT-activating compounds is not an isolated problem, or that the mechanism involved is not contamination of a "foreign" compound, but rather is a consequence of compounds or processes used normally in the manufacture of 5-A-RU. It is paramount that further investigation is carried out to understand and identify the source of NKT cell activity as it is possible that in other laboratories similar activities are being induced using compounds they expect to be devoid of this activity.

Interestingly, when 5-A-RU was filtered through a 0.1μM filter before injection into mice, no loss in human MAIT cell activation was observed, but the filtrate failed to activate DCs or NKT cells *in vivo*. Importantly, filtration of 5-A-RU in a collaborator's lab matched the observations seen in this thesis (Salio, *personal communication*). These data support the hypothesis that particulate matter is inducing NKT cell activity, and hence is responsible for the adjuvant activity of unfiltered 5-A-RU. There are some bodies of research detailing the ability of nanoparticles (NP) to modulate immune responses. When murine bone marrow-derived DCs (BMDCs) were incubated with carbon black NPs, upregulation of CD86 and enhanced allogenic mixed lymphocyte reaction were

observed[269]. Similar results were seen when BMDCs were incubated with TiO_2 NPs, which increased expression of CD86, CD80 and MHC-II, and induced release of TNFα, indicative of activation[270]. Additionally, silica NPs showed similar results *in vitro*, which was reported to be mediated by activation of the NLRP3 inflammasome in BMDCs[271]. Together, these studies indicate that particulate matter has the ability to influence DC activation status, which may contribute to downstream T cell responses. The fact that silica NPs activated DCs is of particular interest, as silica is a common material used in the stationary phase of HPLC columns. It could be speculated that some silica material was contaminating preparations of 5-A-RU and causing the observed activation of DCs *in vivo*. The initial 5-A-RU preparations were not purified by HPLC prior to use, but the compounds used in manufacture were exposed to silica. The hypothesis that particles contributed to the NKT cell responses is hard to align with the published work on NPs, which was mostly conducted on isolated BMDCs *in vitro* and the role of specific cell subtypes were not investigated in the *in vivo* models. However, a recent publication may provide one explanation, where ER stress was shown to induce activation of NKT cells by inducing presentation of stimulatory self-lipids. In the reported study, a small inhibitor of ER Ca^{2+} ATPase, thapsigargan, induced ER stress when incubated with BMDCs[272]. As a consequence, the lipid composition of the BMDCs was altered, and when co-cultured with NKT cells, induced their activation in a CD1d-dependent manner. Numerous studies detail the ability of NPs, including silica, to induce ER stress[273–276]. Thus, it would be necessary in future work to understand the nature of the particulate matter if it is modulating cellular metabolism such through ER-stress.

A known feature of lipids is their proclivity to form large hydrophobic aggregates in polar solvents. Preparation of 5-A-RU for use *in vivo* is carried out in aqueous solution and is further diluted in PBS in order to be injected into mice. Therefore, it is possible that trace amounts of aggregates containing NKT activity could be present in these preparations. It is therefore possible that it was these lipid aggregates that were removed by filtration (and fractionation), but further work is needed to confirm this. Another possibility is that NKT cell-activating compounds adhere to the filter. However, lipids like α-GalCer were not expected to adhere to the polyvinyl fluoride filters used, and experiments by a laboratory colleague have confirmed that soluble α-GalCer will pass through. Nonetheless, α-GalCer aggregates can form in aqueous solution, and can be filtered out if of sufficient size

(Authier-Hall *personal communication*). Further work is required to examine the size and character of the material collected by filtration from the early 5-A-RU preparations.

Loss in NKT cell-based activity of 5-A-RU after filtration suggests a microparticle contaminant. Transmission electron microscopy (TEM) of the filter membrane could aid in visually identifying microparticles and combined with scanning electron microscopy (SEM), and Energy Dispersive X-Ray Spectroscopy (EDS) could identify the elemental composition of the microparticles (i.e. organic/ inorganic material). EDS is of particular interest as it could confirm the presence of inorganic elements such as silica within the 5-A-RU preparations. Furthermore, identification of organic elements would support the notion that the particle may be CD1d-binding ligands. To further scrutinise this possibility, an attempt to form crystal structures with CD1d could be employed in order to identify the structure of the contaminant by X-ray crystallography. However, crystallization requires highly pure samples in order to achieve high resolution, and it is currently unknown whether NKT cell-based activity from these 5-A-RU preparations is derived from a single compound.

One of the final experiments in this chapter was an attempt to establish whether "clean" 5-A-RU does have any activity in the absence of NKT cells by injecting WT or $CD1^{-/-}$ mice with 5-A-RU +/- MG. Interestingly, within the lungs of $CD1^{-/-}$ mice, both cDC-1 and cDC-2 populations showed increased expression of CD86, indicative of activation. This observation highlights how the early experimental results examining MAIT cell activity with the initial 5-A-RU preparations were obscured by the NKT cell activating contaminant. In addition, the earlier studies showed that prior mixing with MG was dispensable for inducing DC activation despite the inclusion of this step being responsible for a much higher level of MAIT cell activation. In fact, due to the apparent lack of requirement of MG in this system, further experiments did not include the use of MG and, therefore, may have failed to identify true MR1-dependent responses. Moreover, the studies of 5-A-RU^{OVA} have to be now reconsidered as newly synthesised 'clean' 5-A-RU was not a strong adjuvant on its own. Due to time constraints, investigation on whether 'clean' 5-A-RU^{OVA} would still result in some generation of antigen-specific responses could not be investigated. However, in $CD1^{-/-}$ mice that lacked NKT cells but not MAIT cells this compound failed to induce DC activation or produce a cytotoxic $CD8^{+}$ T cell response suggesting that MAIT cells were unlikely able to adjuvant antigen-specific

responses when activated with by 5-A-RU^{OVA}. The requirement to form 5-OP-RU before injection to achieve DC activation within the lung suggests that the limiting step in using 5-A-RU as an adjuvant *in vivo* is in formation of the adduct with cellular MG. This would indicate that perhaps a 5-OP-RU-peptide conjugate would more likely drive DC maturation and the initiation of peptide-specific T cell responses. Chemical strategies to do this are currently being explored.

In light of the confounding impact of contaminants in 5-A-RU and related products used in this chapter, the next chapter will detail the use of newly synthesised and 'clean' 5-A-RU in order to "re-investigate" whether MAIT cells can adjuvant DC and T cell responses.

Chapter 5: MAIT cells stimulate the activation of lung resident DC populations

5.1 Introduction

It has now been established that i.v. injection with 5-A-RU +/- MG could lead to significant activation of MAIT cells at systemic sites, namely the lung, liver and spleen. In the previous chapter, improvements in chemical synthesis and preparation of 5-A-RU resulted in a 'clean' product devoid of the NKT cell activity that had been seen in earlier preparations. Furthermore, with this new material, mice treated with 5-A-RU alone did not show clear evidence of DC maturation, but when mixed before injection with MG to form 5-OP-RU, induced significant maturation of lung DC populations in a CD1d-independent manner. The lack of DC maturation with 5-A-RU alone was assumed to be due to its lower potential to activate MAIT cells when compared to injection with the fully-formed agonist, potentially because the availability of MG was limiting *in vivo*. Because synthetically prepared 5-OP-RU was not yet available to the laboratory, further studies on the impact of MAIT cell activation *in vivo* were conducted with 5-A-RU mixed with MG to form 5-OP-RU.

The original hypothesis that MAIT cells can adjuvant T cell responses through the maturation of DCs remained untested due to the confounding contamination issue. It was, therefore, necessary to explore this again with the new, purified 5-A-RU in combination with MG. Thus, this chapter describes experiments aimed to address the remaining questions posed by the previous chapter about the extent to which MAIT cells and DC cross-talk, and how this affects adaptive T cell-mediated immune responses.

5.2 Aims

This thesis aimed to determine whether MAIT cells activated with freshly prepared 5-OP-RU were able to act as cellular adjuvants to induce maturation of DCs and enhance their ability to generate adaptive T cell responses. Unfortunately, in the last chapter, the true influence of MAIT cells on T cell responses was obscured by an unknown NKT cell activating contaminant. Improvements in quality control have yielded 'clean' 5-A-RU that can mediate DC maturation within the lung in a CD1d-independent manner. Therefore, the overall aim of this chapter was to critically analyse the role of MAIT cells in this response and whether the influence of activated MAIT cells is sufficient to help drive T cell responses.

Specifically, this chapter will address:

- The impact of activating MR1-restricted MAIT cells in the lung following i.v. with 5-A-RU + MG
- The impact of activating MAIT cells in vivo on DCs and other immune cell populations
- The ability of MAIT cells to act as cellular adjuvants to stimulate T cell responses to soluble antigen

5.3 Results

5.3.1 Maturation of murine MAIT cells in vivo leads to maturation of DCs in the lung but not the liver or spleen

In the previous chapter, it was shown that 5-A-RU could be filtered to eliminate any NKT-mediated activity *in vivo* while maintaining MAIT cell activity. With the new 'clean' 5-A-RU, it was also shown that when first admixed with MG, 5-A-RU could indeed activate DCs within the lung in a CD1d-independent manner but not within the liver or the spleen.

In order to confirm that this DC maturation was mediated through MAIT cells, 5-A-RU + MG was administered to C57Bl/6J and MR1$^{-/-}$ mice, which do not harbour MAIT cells. To improve the analysis on the maturation of DCs in this model, other co-stimulatory markers, CD80, CD40 and PD-L1 were added to the flow cytometry panel. After administration of the MAIT cell agonist, both cDC-1 and cDC-2 populations in the lung showed increased expression of CD40, CD80, CD86 and PDL-1, which was not seen in MR1-deficient animals (Fig 5.1), confirming the role of MAIT cells in this response.

In the steady-state migratory DC populations traffic from the lung to the mediastinal lymph node as part of homeostatic immune-surveillance[277]. During infection, the number of migratory DCs carrying antigen entering the lung draining lymph node greatly increases, contributing towards the priming of CD8+ T cell responses[277–279]. Therefore, it was next examined whether DCs migrate to the mediastinal lymph node in a similar manner when lung resident MAIT cells were activated with 5-A-RU + MG. Migratory DCs that enter the lymph node can be identified by their increased expression of MHC-II, due to immunogenic interactions in the periphery upon antigen encounter[59]. Therefore, to discriminate between migratory and residential DCs in the lymph node, expression of CD11c accompanied by high MHC-II expression was used as a marker of migratory DC while intermediate MHC-II expression was used to identify resident DCs. Analyses conducted 18 h after administration of the MAIT agonist showed an increase in migratory cDC1 and cDC2s infiltrating the mediastinal lymph nodes of treated wild-type mice, as seen by the increased number and frequency of MHC-II^{hi}CD11c^{int} cells (Fig 5.2a & c). This migration was not observed in MR1-deficient mice, so was dependent on activation of MAIT cells. In addition to this increased frequency, which was also observed in the lung, the migratory DCs showed increased expression in maturation marker CD86 (Fig

2c). In contrast, the resident cDC1 and cDC2 populations did not exhibit any increase in maturation (Fig 5.2b).

In summary, these results demonstrate that MAIT cells are poised to interact with DCs in the lung resulting in their maturation and migration into the draining LN. It is also now clear that MAIT cells are also unable to interact with DCs in the liver and the spleen in a similar manner which may be reflective of differences in population frequency or tissue-specific functionality of MAIT cells.

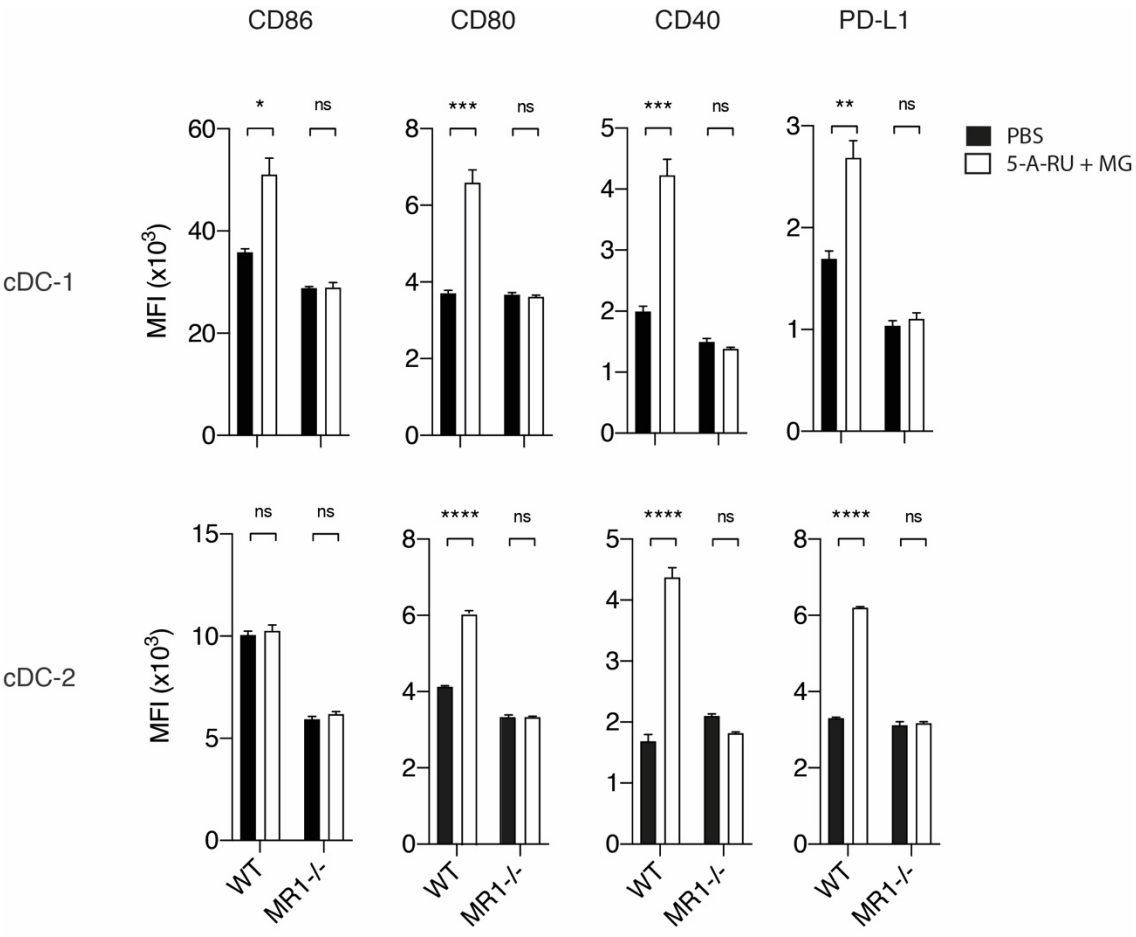


Figure 5.1. MR1-dependent maturation of lung resident DCs. WT or MR1$^{-/-}$ mice (n=5) were administered i.v. PBS or 5-A-RU + MG. After 18 h, lungs were harvested and stained for flow cytometry. MFI was calculated for CD86, CD80, CD40 and PDL-1 on lung cDC-1 and cDC-2. Data presented is representative of two independent experiments. Graphs are depicted with the mean ± SEM. Statistical significance was determined by Two-Way ANOVA with Tukey's multiple comparisons test.

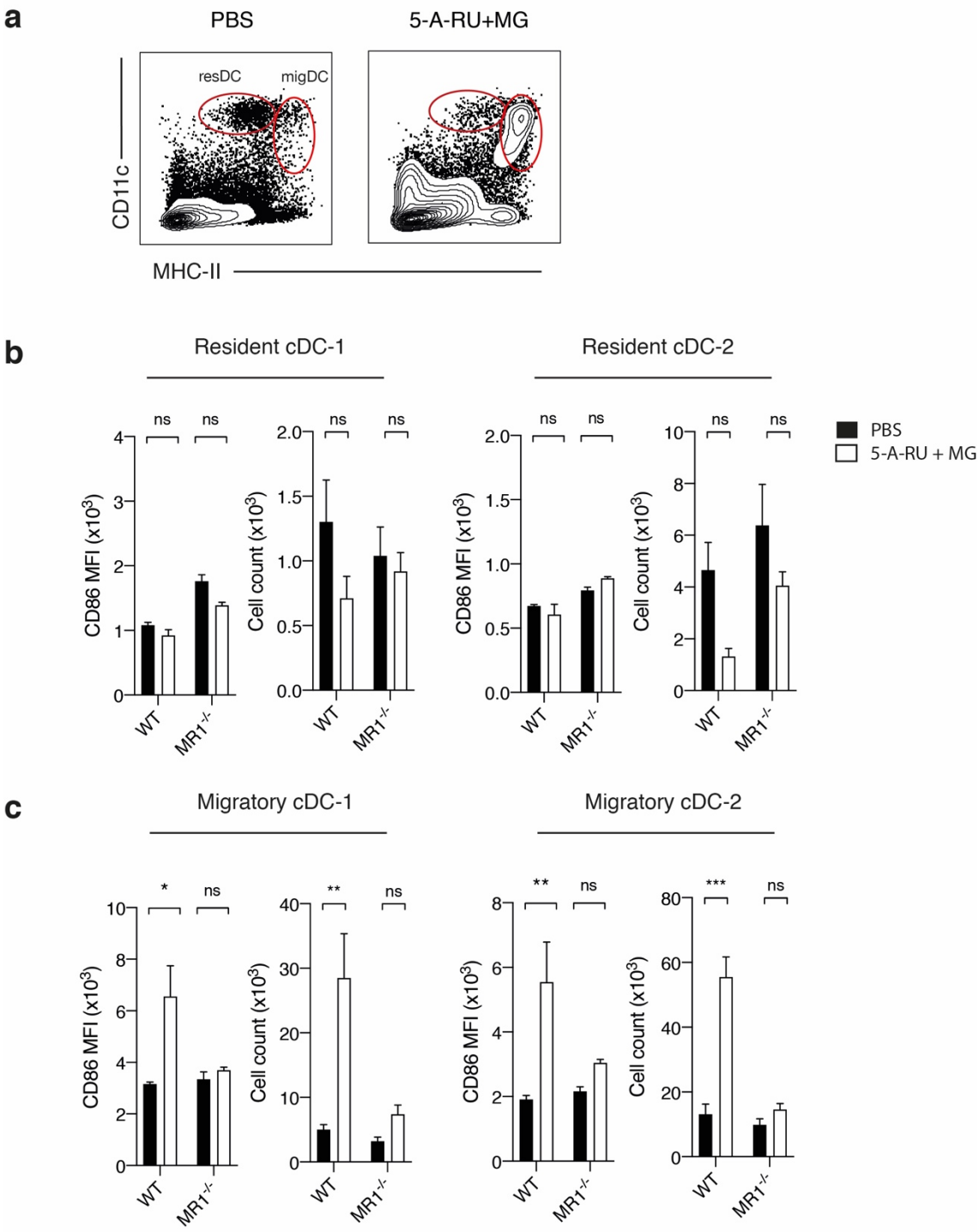


Figure 5.2. Activated MAIT cells mediate accumulation of activated migratory DCs within the mediastinal lymph node. WT or MR1$^{-/-}$ mice (n=5) were administered i.v. PBS or 5-A-RU + MG. After 18 h, mediastinal lymph nodes were harvested and stained for flow cytometry. (a) Resident (res) DC and migratory (mig) DC populations were gated according to their CD11c and MHCII expression from CD64^{-} B220^{-} cells. MFI was calculated for CD86, CD80, CD40 and PDL-1 on lung cDC-1 and cDC-2 and cell number enumerated from either resident (b) or migratory DCs (c). Data presented is representative of two independent experiments. Graphs are depicted with the mean ± SEM. Statistical significance was determined by Two-Way ANOVA with Tukey's multiple comparisons test.

5.3.2 Activation of MAIT cells leads to infiltration of other myeloid cells in the lung

One of the hallmarks of early inflammation is the influx, activation and differentiation of innate-immune cells to the site of infection through the production of local inflammatory cytokines and chemokines. Indeed, MAIT cells have already been shown to be able to produce GM-CSF in response to pulmonary bacterial infection, which leads to the infiltration and differentiation of inflammatory monocytes[27]. When assessing DC maturation in response to 5-OP-RU in the previous experiments, it was notable that infiltration of other inflammatory populations was observed. Therefore, to analyse these cells in more detail a previously published gating strategy was used to allow for the detection of neutrophils, eosinophils and inflammatory monocytes[280] (Fig 5.3a). When mice were administered 5-A-RU + MG, after 18 h there was an increase in the number and frequency of $Ly6C^+$ inflammatory monocytes, neutrophils and eosinophils (although the frequency of eosinophils did not reach statistical significance) which was not present in $MR1^{-/-}$ mice (Fig 5.3b & c). Therefore, this data demonstrates that MAIT cells are able to influence the infiltration or expansion of myeloid populations within the lungs.

Previous research has shown that the presence of MAIT cells during infection is important in the generation of pro-inflammatory cytokines that conferred protection[26,187]. Moreover, upon *ex vivo* stimulation, MAITs are potent producers of cytokines[23,25]; however, their ability to influence cytokine responses upon *in vivo* administration of 5-OP-RU has not been defined. Importantly, as the relationship between expression of specific cytokines has been correlated with the influx of defined cell populations, analysis on the cytokine pattern post 5-OP-RU injection *in vivo* could help to inform possible new cell interactions with MAIT cells. To address this, serum from mice was obtained 6 h after i.v. with 5-A-RU + MG. Pam2Cys, a potent TLR2 agonist, was utilised as a positive control due to its potent properties in inducing systemic cytokine release. In order to analyse the cytokines, a 23-plex cytokine bead array was used. Within this kit, specific antibodies towards each of the 23 cytokines are coated onto beads with known fluorescent indexes. Therefore, this method allows for the simultaneous capture and differentiation of all 23 selected cytokines within the analyte and quantification by measuring their unique fluorescence intensity. Of the cytokines analysed (Appendix D) an increase in circulating IL-5 and granulocyte-colony stimulating factor (G-CSF) was observed after administration of 5-A-RU + MG (Fig 5.4).

Collectively, these results demonstrate that activated MAIT cells can influence the lung immune microenvironment through recruitment, expansion or activation of other myeloid populations. Whether MAIT cells are directly producing cytokines such as G-CSF and IL-5 in response to 5-A-RU + MG or are mediating cross-activation of other immune subsets, is yet to be determined but warrants further investigation.

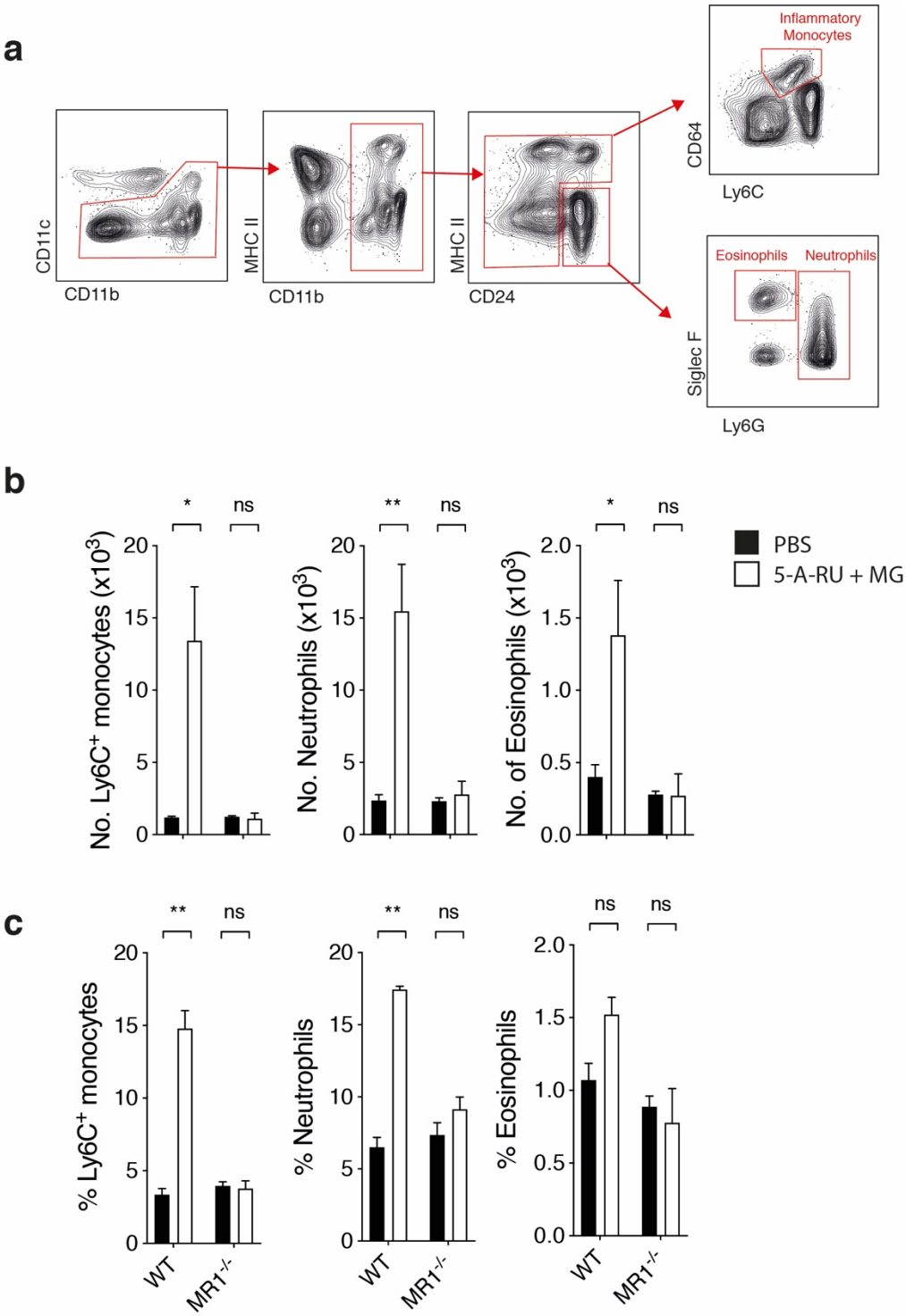


Figure 5.3. Activated MAIT cells results in increased myeloid populations in the lung. (a) Representative flow cytometry gating strategy to detect monocytes, eosinophils and neutrophils. Cells were gated first for singlets, live cells and were CD45.2$^+$. Mice (n=5) were injected with PBS or 5-A-RU + MG. After 18 h lungs were harvested and assessed by flow cytometry. The total number of Ly6C+ monocytes, neutrophils and eosinophils were enumerated from the lung (b) and the frequency as a percentage of live CD45.2$^+$ cells calculated (c). Data presented is representative of two independent experiments. Graphs are depicted with the mean ± SEM. Statistical significance was determined by Two-Way ANOVA with Tukey's multiple comparisons test.

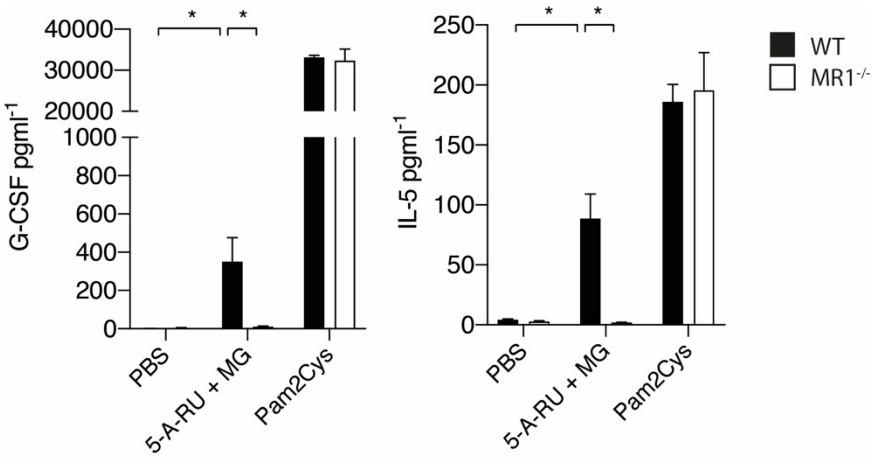


Figure 5.4. MR1-dependent increase in systemic G-CSF and IL-5. Mice (n=5) were administered i.v. PBS, 5-A-RU + MG or Pam2Cys and bled after 6 h for serum. Serum was analysed by Bioplex. Data presented is representative of a single experiment. Graphs are depicted with the mean ± SEM. Statistical significance was determined by Two-Way ANOVA with Tukey's multiple comparisons test.

5.3.3 MAIT cell-mediated maturation of DCs is insufficient to help drive T cell responses

The results so far have indicated that MAIT cells have the capacity to induce the maturation and migration of lung DCs and influence other lung-resident myeloid populations. These data indicated that MAIT cells could potentially be able to drive the formation of adaptive T cell responses if co-administered with antigen. Therefore, to test this hypothesis, mice were administered 5-A-RU + MG i.v. in combination with OVA and one week later, the expansion of OVA-specific $CD8^+$ T cells within the lungs and spleen was assessed using an OVA_{257-64} (SIINFEKL)-loaded H-$2K^b$ pentamer. As a positive control, OVA was injected with the TLR2 agonist Pam2Cys, which as expected, led to increased numbers of OVA-specific $CD8^+$ T cells in lung and spleen relative to vehicle-treated controls. In contrast, co-administration of OVA with 5-A-RU + MG did not increase levels of OVA-specific $CD8^+$ T cells (Fig 5.5a).

This data suggests that MAIT cell activation with 5-OP-RU is not sufficient to adjuvant the induction of $CD8^+$ T cell responses to soluble antigens. To further interrogate this in a more sensitive system, mice were administered a small population of $CD8^+$ T cells from OT-I mice. These mice express transgenic TCRs specific for $OVA_{257-264}$, a $CD8^+$ T cell epitope that binds to H-$2K^b$. With this method, flow cytometry could be used to identify the expansion of this OVA-specific population over time. The OT-I strain and C57BL/6J hosts used in this experiment were also congenic for CD45, with OT-I mice expressing the CD45.1 isoform, and C57BL/6 mice the CD45.2 isoform; the transgenic OVA-specific $CD8^+$ T cells could, therefore, be identified through the use of antibodies for the TCR rearrangement used in the transgene (Vα2) and expression of CD45.1.

After receiving donor cells intravenously, mice were administered 5-A-RU + MG + OVA, or α-GalCer + OVA as a positive control which is known to induce a significant T cell response[19]. The expansion of OVA-specific T cells was followed over 10 d in the blood (Fig 5.5b). As expected, administration of OVA with α-GalCer lead to a significant expansion of the OT-I population. In contrast, no increase in OT-I cell numbers was observed following administration of OVA and 5-A-RU/MG. The MAIT cell-mediated maturation of DCs in the lung is therefore insufficient to help drive conventional $CD8^+$ T cell responses to simultaneously administered antigens.

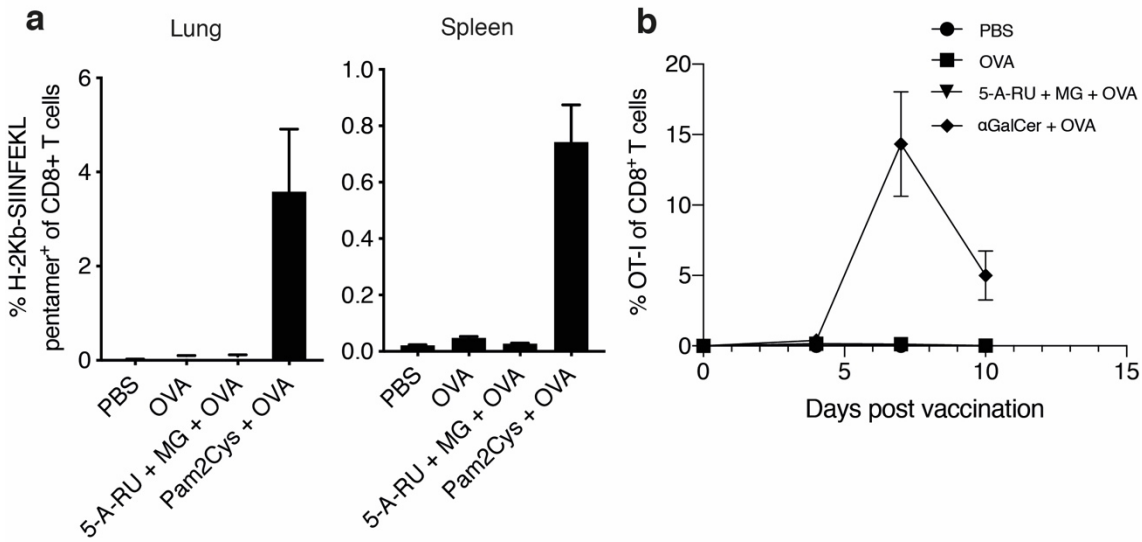


Figure 5.5. MAIT cells are insufficient to drive T cell responses to OVA. (a) Mice (n=3) were administered i.v. PBS, OVA or OVA combined with either 5-A-RU + MG or Pam2Cys. After seven days, lungs and spleens were harvested for flow cytometry. Graph depicts the frequency of OVA-specific CD8+ T cells by staining with an H-2Kb-SIINFEKL pentamer from CD45+ CD64- B220- CD3+ CD8+ cells. (b) Mice (n=5) were adoptively transferred CD8+ OT-I cells and after 24 h intravenously administered PBS, OVA or OVA combined with either 5-A-RU + MG or αGalCer. At d 4,7 and 10, mice were bled and the number of OT-I CD8+ T cell was assessed by flow cytometry. OT-I cells were defined as B220- CD3+ CD45.1+ CD8+ Vα2+ cells. Data presented is representative of two independent experiments. Graphs are depicted with the mean ± SEM.

5.3.4 Activation of MAIT cells leads to specific IL-12 production in lung DCs

The heterodimeric cytokine IL-12p70, formed by covalently linked p35 and p40 subunits, is an essential polarizing signal that drives the differentiation of NK cells, Th1 cells and importantly the generation of CTLs[281]. Production of IL-12 by DCs, specifically cDC1s, are critical in the formation of adaptive $CD8^+$ T cell responses. Given MAIT cells can induce DC maturation but do not appear to be able to adjuvant peptide-specific T cell responses it was next investigated whether MAIT cells would be able to provide appropriate stimuli to DCs in order to produce bioactive IL-12p70. To this end, C57BL/6 or MR1-deficient mice were treated with 5-A-RU + MG, or Pam2Cys as a positive control, and bled after 6h to examine IL-12 within the circulation. No IL-12p70 or the IL-12p40 subunit could be found in either mouse strain treated with 5-A-RU + MG (Fig 5.6a), whereas Pam2Cys induced significant levels. It could be reasoned that due to the lack of DC maturation seen at sites other than the lung, that perhaps IL-12 was produced only within the lungs and did not reach systemic circulation, and perhaps was sequestered within the local tissue environment. To explore this possibility, IL-12p40^{eYFP} mice were used which contain a knock-in IL-12p40 allele linked to an enhanced yellow fluorescent protein (eYFP), so that cells expressing IL-12p40 will also produce eYFP. This can be detected by flow cytometry, allowing identification of tissue-specific IL-12 production *in situ*. These mice were injected i.v. with either 5-A-RU + MG or α-GalCer which has been shown *in vivo* to be a potent inducer of IL-12 production by DCs. The cDC2 population of these mice showed no significant upregulation of IL-12p40 in any of the treatment groups (data not shown), consistent with reports detailing that IL-12 is prominently produced by cDC1s[282]. Within the lungs, administration of 5-A-RU + MG led to a significant increase in IL-12p40 production, even more so than α-GalCer (Fig 5.6b). In line with the lack of DC maturation in the liver or spleen, 5-A-RU + MG did not induce significant production of IL-12p40 at these sites, whereas significant levels were seen in mice treated with α-GalCer. Together these results align with the earlier findings that MAIT cell-mediated priming of DCs within the tissues investigated were restricted to the lung.

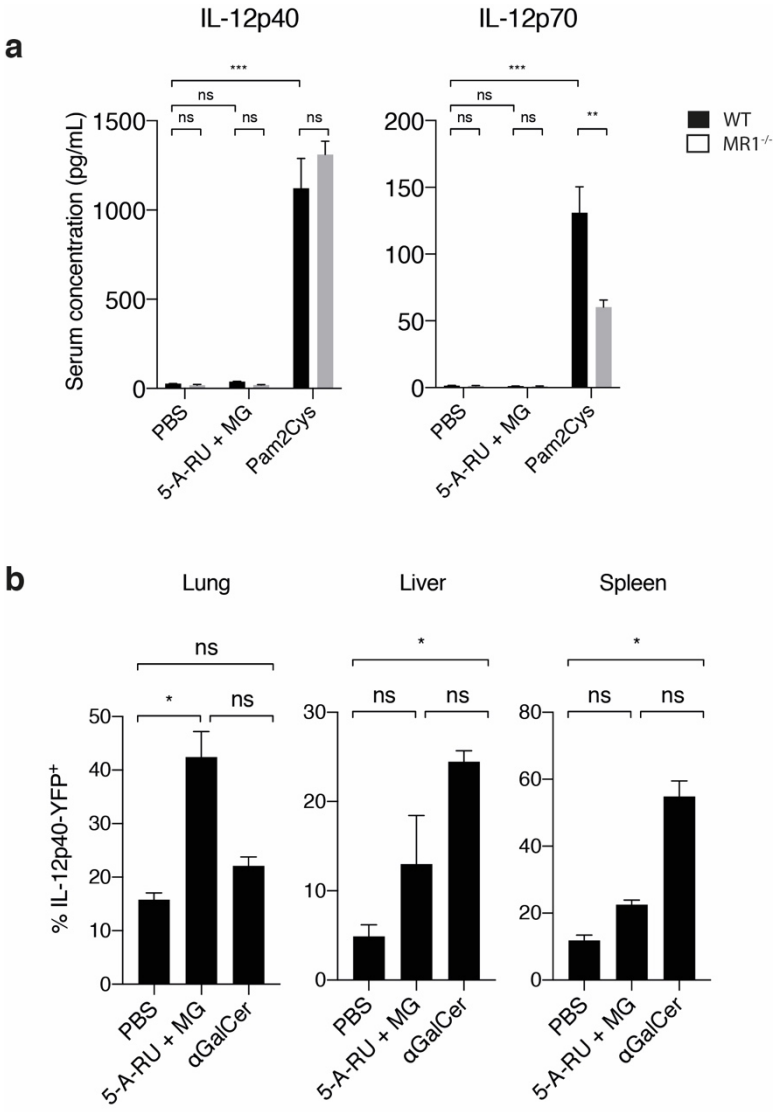


Figure 5.6. Tissue-specific induction of IL-12 by MAIT cells. (a) Mice (n=5) were injected with 5-A-RU + MG or Pam2Cys i.v. and bled after 6 h for serum. Serum was analysed by bioplex to assess IL-12p40 and IL-12p70. (b) IL-12p40^{eYFP} mice (n=3) were injected with 5-A-RU + MG or αGalCer i.v. Lungs, livers and spleen were harvested after 18 h and eYFP expression was assessed by flow cytometry on cDC-1 cells. Data presented in (a) is from a single experiment and (b) is representative of two independent experiments. Graphs are depicted with the mean ± SEM. For (a) Statistical significance was determined by Two-Way ANOVA with Tukey's multiple comparisons test and (b) was determined by One-Way ANOVA with Tukey's multiple comparisons test.

5.3.5 CD40L is upregulated on activated MAIT cells but may be dispensable in MR1-dependent maturation of DCs

Ligation of CD40 on human and murine DCs results in increased expression of MHC molecules and co-stimulatory molecules and enhances production of bioactive IL-12[15,16,18]. This licensing of DCs is particularly important in the priming of $CD8^+$ T cells to induce functional CTL. Stimulation of the CD40 on DCs can be accomplished by $CD40L^+$ T helper cells, typically $CD4^+$ T cells, although innate-like T cells such as NKT cells can also efficiently perform this role[15,16,283]. Because MAIT cells can express CD40L and have been shown to activate human DCs in a CD40-dependent fashion *in vitro*[29], it was possible that the lack of T cell response to co-administration of antigen with the MAIT agonist in earlier experiments could be caused in a failure of MAIT cells to interact with DCs via CD40-CD40L interactions *in vivo*.

To test this, it was first necessary to establish whether MAIT cells *in vivo* upregulate CD40L in response to stimulation. Mice were administered PBS or 5-A-RU + MG, and the levels of CD40L were measured after 3 h. In line with *in vitro* findings for human MAIT cells[29], lung resident MAIT cells indeed upregulated CD40L in response to 5-A-RU + MG (Fig 5.7a). To assess the role of CD40L-CD40 engagement in MAIT-cell mediated DC maturation, mice were administered a CD40L-blocking monoclonal antibody (αCD40L) 1 d before, and the day of, i.v. administration with 5-A-RU + MG. To show that the blocking strategy worked, the same regimen of antibody treatment was used in combination with i.v. administration of α-GalCer, as NKT cell-mediated DC maturation is known to involve CD40-signalling. After 18 h, NKT cell-mediated maturation of DCs was observed in the lung of α-GalCer-treated mice, with upregulation of CD86, CD80 and CD40 and PD-L1. Interestingly, upregulation of some, but not all, of these markers was blocked in animals treated with αCD40L, notably CD40 (on cDC1) and CD86 (on cDC1 and cDC2). In contrast, MAIT-cell-mediated maturation of DCs was not blocked by αCD40L antibody, except CD40 upregulation on cDC2 (Fig 5.7b). Although not definitive, these results would indicate that CD40L may not be a primary mechanism in which MAIT cells induce DC maturation and may be why 5-OP-RU is unable to induce T cell responses in combination with antigen.

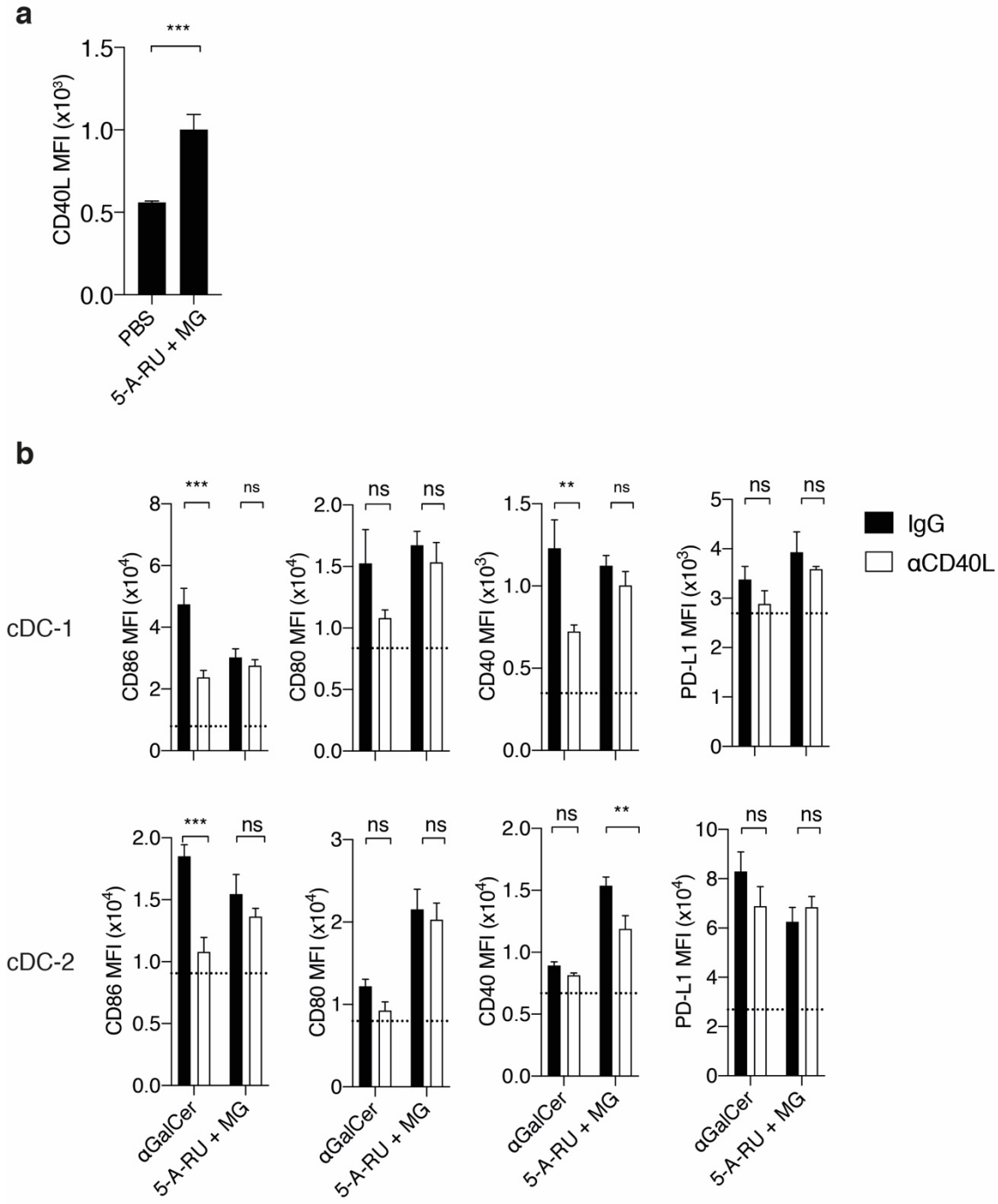


Figure 5.7. Upon activation MAIT cells upregulate CD40L but is dispensable in the maturation of DCs. (a) Mice (n=5) were administered i.v. PBS or 5-A-RU + MG. After 3 h lungs were harvested and MAIT cells were assessed for upregulation of CD40L and MFI was calculated by flow cytometry (b) Mice (n=5) were administered intraperitoneally αCD40L antibody or Armenian Hamster IgG as control the day before and the day of i.v. with PBS, 5-A-RU + MG or αGalCer. After 18 h, lungs were harvested and stained for flow cytometry and MFI was calculated for CD86, CD80, CD40 and PDL-1 on lung cDC-1 or cDC-2. Average of PBS MFI for each marker is represented by the dotted line. Graphs are depicted with the mean ± SEM. Statistical significance for (a) was determined by t-test and (b) was by Two-Way ANOVA with Tukey's multiple comparisons test.

5.3.6 Role of type I interferon signalling in the response to 5-A-RU + MG

As CD40-CD40L ligation did not appear to be critical in the MAIT-dependent DC maturation seen, it was possible that MAIT cells mediated DC maturation through a contact independent manner mediated by cytokines. Type I IFN are known as activatory cytokines that can drive the maturation and differentiation of DCs through binding to the IFN α/β receptor (IFNAR) on the cell surface.[90,284,285]. Thus, it was determined whether type I IFNs signalling played a role in MAIT cell-mediated DC maturation in the lungs.

One way to examine whether type I IFNs was involved in MAIT cell-mediated DC maturation was to look for expression of markers regulated by IFN regulatory factors. Bone marrow stromal Ag 2 (BST-2), a transmembrane protein that prevents virus release from infected cells, is one such marker. Importantly, the BST-2 promotor contains an IFN regulatory factor binding site, and as a consequence BST2 expression upregulated upon many different immune cell-types after exposure to type I IFNs, including DCs[286]. Therefore, expression of BST-2 was examined on DCs after administration of 5-A-RU + MG. As a positive control for this experiment, the TLR3 agonist, poly I:C, was used as TLR3 is highly expressed on pDCs, a cell-type that produces marked amounts of type I IFNs upon stimulation. Interestingly, mice treated with 5-A-RU + MG showed an increased expression of BST-2 on lung cDC2, and a trend towards increased expression on cDC1 that did not reach significance (Fig 5.8a). Expression of BST-2 on these DCs indicates the exposure to type I IFNs in this model. One possibility is that MAIT cells could be directly reacting to 5-OP-RU loaded on MR1 on pDCs, which are potent producers of type I IFN. Therefore, the maturation status of pDCs was assessed in the lungs by gating on $CD11c^+$ $B220^+$ cells that express the pDC-associated marker $SiglecH^+$ cells (after excluding $CD64^+$ macrophages) and examining CD86 expression. There was a minor but significant increase in pDC maturation in response to 5-A-RU + MG (Fig 5.8b). These results collectively suggest that type I IFNs are involved in MAIT cell-mediated maturation of cDCs, and that this may be stimulating IFN production by activating pDCs.

To assess the extent in which Type I IFNs are involved in driving DC maturation, mice were treated with an antibody which blocks IFNAR on d -1 and d 0 in order to prevent binding of IFNα or β. On d0, mice were also treated with 5-A-RU + MG, and after 18 h,

the level of DC maturation was measured. Again, Poly I:C was used as a positive control as a known inducer of type I IFN, which could be significantly blocked by αIFNAR pre-treatment (Fig 5.9). In contrast, type I IFN signalling seemed to be dispensable in mice treated with 5-A-RU + MG. With the exception for minor decreases in CD40 expression and PDL-1 expression in DC-2, the DC maturation by 5-A-RU + MG was largely unaffected by αIFNAR treatment. Collectively, this data shows that *in vivo* administration of 5-A-RU + MG does result in some type I IFN signalling. However, these signals were not the major drivers of DC maturation.

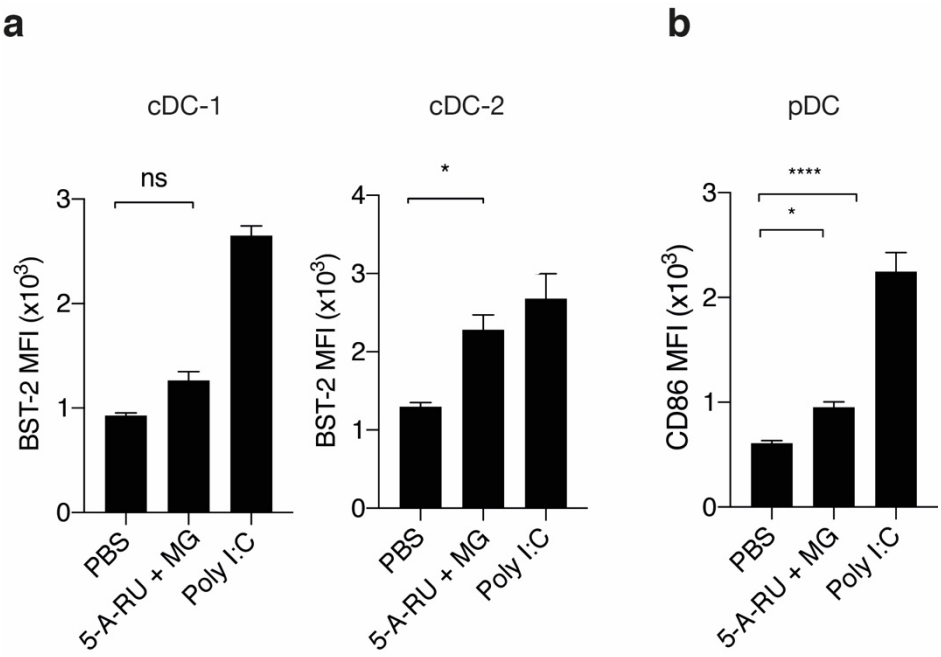


Figure 5.8. BST-2 upregulation of DCs and maturation of pDCs. Mice (n=5) were administered i.v. PBS or 5-A-RU + MG. After 18 h, lungs were harvested and stained for flow cytometry. (a) MFI for BST-2 was calculated on cDC-1 and cDC-2 and CD86 was measured on pDCs identified as $CD11c^+$ $SiglecH^+$ cells from $B220^+$ $CD64^-$ cells. Data presented is from a single experiment. Graphs are depicted with the mean ± SEM. Statistical significance was determined by One-Way ANOVA with Tukey's multiple comparisons test.

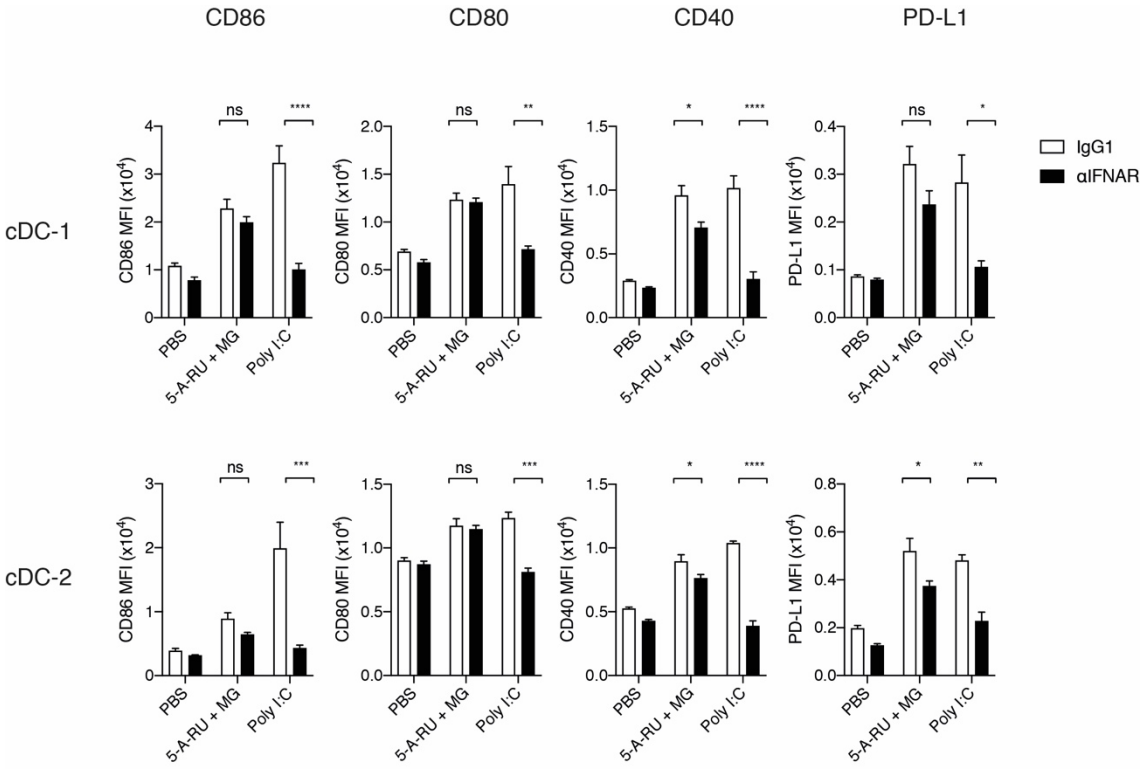


Figure 5.9. Type I IFN signalling is not crucial in MAIT-dependent DC maturation in the lung. Mice (n=5) were administered intraperitoneally αIFNAR antibody or Rat IgG as control on d -1 and d0. On d0 mice were administered PBS, 5-A-RU + MG or Poly I:C i.v. After 18 h, lungs were harvested and stained for flow cytometry and MFI was calculated for CD86, CD80, CD40 and PDL-1 on lung cDC-1 or cDC-2. Data presented is from a single experiment. Graphs are depicted with the mean ± SEM. Statistical significance was determined by Two-Way ANOVA with Tukey's multiple comparisons test.

5.3.7 Co-administration of a TLR agonist with 5-A-RU + MG helps drive $CD8^+$ T cell responses

Previous studies conducted on human DCs *in vitro* showed that signals from activated MAIT cells co-operate with stimuli via pathogen-derived pattern recognition receptors to induce release IL-12p70, the bioactive form of IL-12[29]. To determine if a similar cooperative activity was required to improve DC function and support T cell responses *in vivo*, OVA and 5-A-RU + MG was injected with low doses of the TLR2 agonist Pam2Cys. As seen in Fig 5.10a, combining MAIT cell activation with TLR stimulation-induced increased expression of maturation markers CD86, CD40 and PDL-1 on lung DCs, observed 18 h after the administration of the agonists. Using this combination of agonists with administration of OVA protein also resulted in enhanced induction of an OVA_{257-64}-specific $CD8^+$ T cell response compared to when OVA was injected with 5-A-RU/MG or Pam2Cys alone. These responses were observed without transfer of OT-I cells, so represent activated cells from the endogenous T cell repertoire. The enhanced responses in lung and liver after 7 d were statistically significant, whereas the slight improvement in spleen did not reach significance. Critically, the impact of including 5-A-RU + MG was abrogated in MR1-deficient animals, highlighting the role of MAIT cells in the response (Fig 5.10b). Therefore, given the correct additional signals, MAIT cells can contribute to the induction of T cell responses to soluble antigens.

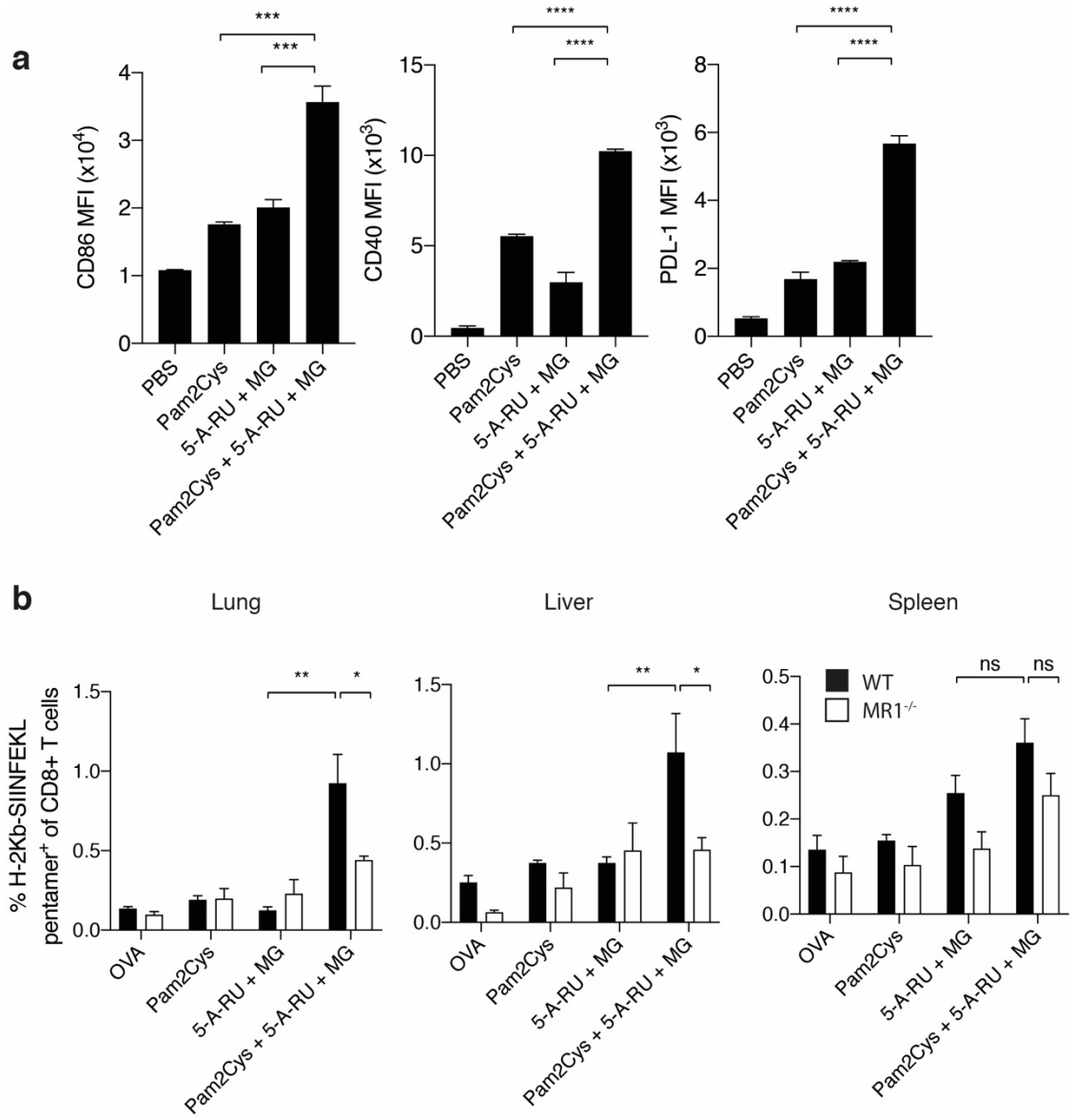


Figure 5.10. MAIT cells can co-operate with TLR signalling to induce T cell responses to OVA. (a) Mice (n=3) were administered i.v. PBS, 5-A-RU + MG, 375ng of Pam2Cys or combined 5-A-RU + MG and Pam2Cys. After 18 h, lungs were harvested and stained for flow cytometry and MFI was calculated for CD86, CD40 and PD-L1 on all cDCs. (b) WT and MR1$^{-/-}$ mice (n=5) were administered i.v. PBS, OVA or OVA combined with either 5-A-RU + MG, 375ng of Pam2Cys or a combination of 5-A-RU + MG and Pam2Cys. After 7 d, lungs and livers were harvested for flow cytometry. Graph depicts the frequency of OVA-specific CD8+ T cells by staining with an H-2Kb-SIINFEKL pentamer as in Fig 6a. Data presented is representative of two independent experiments. Graphs are depicted with the mean ± SEM. Statistical significance was determined by Two-Way ANOVA with Tukey's multiple comparisons test.

5.4 Discussion

In the previous chapter, it was established that 5-A-RU alone was insufficient to induce maturation of DCs, but could do so if mixed with MG prior to injection to form the active ligand 5-OP-RU. Here, when looking at lung tissue, i.v. injection with 5-OP-RU lead to significant upregulation of co-stimulatory molecules in both cDC-1 and cDC-2 populations (with the exception of CD86 on cDC-2). Importantly, this response was completely dependent on MR1, confirming that MAIT cells were responsible for the cross-talk with DCs *in vivo*. Interestingly, this DC maturation was only seen in the lung, but not in the liver nor the spleen. This result may be reflective of differences in the frequency of MAIT cells between the tissues tested. A previous report showed that lung MAIT cells were the most frequent tissue population in C57BL/6 mice, accounting for ~3% of lung T cells, whereas MAIT cells in the liver and spleen represent only ~0.5% and ~0.06% of T cells, respectively[23]. The number of MAIT cells available to feedback onto DCs may, therefore, be a critical determinant in the observed response. However, the frequency of MAIT cells in our colony of C57BL/6 mice differs from this report, with both the lung and the liver having similar frequencies of around 0.5% of T cells (Appendix B). This would suggest that some tissue-specific functionality of MAIT cells exists that may affect their propensity to interact with DCs. A published microarray analysis of mouse MAIT cells, which included unsupervised clustering, showed that across the lung, liver and spleen, MAIT cells have distinct transcriptional profiles[198], supporting the notion that MAIT cells show tissue-specific functionality. The genes that would distinguish cells with capacity for DC maturation would require further investigation. The dataset from the microarray study is publicly available and could be further analysed to assess any baseline differences between different MAIT cell populations in genes associated with DC cross-talk such as co-stimulatory receptors, cytokines and adhesion molecules. One limitation of this analysis would be that these tissue-specific differences in functionality may only become apparent when MAIT cells become activated, influenced by their tissue microenvironment.

Transport of antigen to draining lymph nodes is an important mechanism in the generation of adaptive immune responses. Tissue-resident DCs that acquire antigens and receive signals to migrate, upregulate key chemokine receptors such as CCR7 in order co-localise and interact with naïve T cells within lymph nodes[59]. Alternatively, molecules can also

directly drain into the lymph nodes independent of cellular uptake. In mice treated with 5-A-RU + MG there was a marked increase of migratory DC in the mLN, which was MR1 dependent. Furthermore, these migratory DCs had a higher maturation status than resident DCs, indicating that MAIT cells within the LN were not interacting with and activating resident DCs but rather maturation occurred through lung-resident MAIT cells that resulted in the trafficking of DCs to the LN. A limitation of these observations, however, is the inability to distinguish between resident and migratory DCs with high specificity in this model. It is possible that DCs within the lymph node did indeed become activated and as a result, upregulated MHC-II and merged with migratory DCs in the flow cytometry gate. Indeed, there was decrease of the number of resident DCs (although statistically insignificant) in mice treated with 5-A-RU + MG, and activated MAIT cells were observed in the mLN after i.v. injection at the same time point (data not shown). Fluorescently-labelled antigens have been used in order to study DCs draining to afferent lymph nodes which clearly distinguishes migratory versus resident populations[287–289]. If the population of migratory DCs observed is solely derived from lung tissue a similar approach with a fluorescently labelled 5-OP-RU would allow accurate identification. Such a compound was not available for the studies here. However, due to 5-OP-RU being a small molecule, attachment of a fluorescent molecule may heavily impact on its biodistribution and the cells which preferentially endocytose it. Alternatively, pertussis toxin could be used to inhibit G protein-coupled migration of DCs into lymph nodes[290] to determine the relative contributions of lung vs mLN resident MAITs in this response.

After injection with 5-A-RU + MG, an increase in the number and frequency of $Ly6C^+$ monocytes could be seen. *In vivo* infection of mice with *F. tularensis* revealed that GM-CSF production by MAIT cells can drive the differentiation of inflammatory monocytes which may contribute to the recruitment of $CD4^+$ T cells in this model[27]. A number of other *in vivo* studies confirm that inflammatory monocytes readily contribute to the recruitment of $CD4^+$ T cell responses during microbial infections[291–294]. Therefore, it is possible that activation of MAIT cells by 5-A-RU + MG could be used to direct the expansion of antigen-specific $CD4^+$ T cells through the recruitment of inflammatory monocytes. Unfortunately, the induction $CD4^+$ T cell responses were not directly measured in this chapter; however, this possibility is currently under investigation.

Analysis of serum cytokines also revealed an increase in the levels of IL-5 and G-CSF, which was followed by an infiltration of eosinophils and neutrophils into the lung tissue. During the course of helminth infections, eosinophils are recruited to the site of infection through Th2-associated cytokines such as IL-4 and IL-13 and the chemokine eotaxin where they are subsequently activated by IL-5. NKT cells can also induce eosinophilia after intranasal administration of α-GalCer[295]. In this model, NKT cells could recruit eosinophils in an IL-4 and IL-13 dependent manner and directly activate them through the production of IL-5. Current literature suggests that MAIT cells are not producers of these Th2 cytokines, evident in their limited expression of GATA3[190]. Production of high levels of IL-13 has been observed in MAIT cells, although only after multiple rounds of stimulation *in vitro*[296]. In the current study, there was no evidence of increased levels of eotaxin, IL-4 or IL-13 in the serum of mice treated with 5-A-RU + MG. However, analysis of cytokines in serum may have limited sensitivity as the observed MAIT cell-mediated responses appear to be isolated to the lung. Therefore, to further these findings analysis of bronchoalveolar lavage (BAL) fluid may reveal some significant differences in cytokine levels after treatment as it would provide a more concentrated and representative sample of the local inflammatory environment.

Neutrophils are key anti-microbial effector cells of the innate immune system that can eliminate bacteria through phagocytosis, the release of anti-microbial proteins (degranulation), or production of neutrophil extracellular traps (NETs)[297]. In addition to these direct anti-microbial functions, neutrophils can also exhibit anti-inflammatory properties. In response to α-GalCer, neutrophils rapidly migrate to sites of NKT-mediated inflammation and directly inhibit their function through cell-cell contact[298]. A recently published paper described this function in human MAIT cells, where neutrophils suppressed MAIT cell function through a process that involved cell-cell contact and was dependent on H_2O_2[299]. Additionally, the authors showed that TNF-production by MAIT cells *in vitro* mediated neutrophil death. A negative feedback loop was proposed in which MAIT cells and neutrophils suppress one another in order to resolve inflammation. Neutrophils also promote tissue repair through the release of IL-22 and promotion of cell proliferation[300]. It is unknown in this current study whether activation of MAIT cells led to significant lung pathology, although no overt changes were observed. It could be hypothesised that neutrophils may be reacting to a chemokine/cytokine gradient as a result of MAIT-mediated inflammation. For example, IL-17, known to be highly

expressed by MAIT cells, induces G-CSF, CXCL1, CXCL2 and CXCL5 production in mouse fibroblasts and epithelial cells which are known to promote the recruitment of neutrophils[301–303].

Having established that MAIT cells were able to induce inflammation within the lung and drive the maturation and migration of DCs, it remained possible that MAIT cells could function as cellular adjuvants to help induce adaptive immune response to antigens. However, when mice were co-administered 5-A-RU + MG and OVA protein, there was no expanded pool of OVA-specific $CD8^+$ T cells detected in the lung or the spleen after 7 d. Furthermore, increasing the sensitivity of detecting a response by adoptively transferring a cohort of naïve transgenic OVA-specific $CD8^+$ T cells before "vaccination" also yielded no results. This result was surprising considering high levels of co-stimulatory markers present on the cDC-1 population within the lung.

It is well established that the CD40-CD40L signalling axis is critically important in driving effective T cell responses. When DCs lack CD40, they are unable to prime $CD4^+$ and $CD8^+$ T cells even when they have high expression of MHC and co-stimulatory markers CD80/86[18]. Importantly, ligation of CD40L to CD40 is critical in 'licensing' DCs to produce IL-12 and effectively engage with naïve $CD8^+$ T cells[15,18,157]. Indeed, it was found that lung MAIT cells were also able to upregulate CD40L in response to injection with 5-A-RU + MG. However, blockade of CD40L did not show any effect on upregulation of any of the co-stimulatory markers in mice treated with 5-A-RU + MG, suggesting a possible fault in the ability of MAIT cells to engage CD40L with CD40 on DCs or that this was not the mechanism by which MAITs promoted DC maturation. The clear limitation of these observations is that only CD86 and CD40 expression was significantly down regulated by CD40L blockade in α-GalCer treated mice, a treatment that is known to enhance T cell responses via CD40-signalling, and therefore acted as the positive control. In order to definitively assess the role of CD40L in this model, it would be relevant to examine the effect of CD40L blockade further upstream of the response, such as in the ability to produce IL-12 (which is known to be modulated by CD40-signalling). To improve upon the antibody blocking method used, detection of CD40L expression by flow cytometry could be used to confirm blockade of this molecule, as blockade would obscure binding of fluorescently labelled CD40L (of the same clone).

Alternatively, use of CD40L deficient mice would overcome antibody blocking challenges.

As DC maturation by MAIT cells did not seem to require CD40L signalling, this prompted an investigation in whether MAIT cell-mediated maturation was mediated by soluble factors such as cytokines which are also known to be key drivers of DC maturation. Type I interferons, i.e. IFNα/β, are known to drive DC maturation[285]. There was some indication that type I IFN signalling was induced by administration of 5-A-RU + MG as cDC-1s showed a modest upregulation of the type I IFN-inducible marker BST-2. Plasmacytoid DCs are one of the main producers of these IFNs, and after treatment with 5-A-RU + MG showed a marginal level of maturation. This observation aligns with results seen *in vitro* showing, that human MAIT cells could activate pDCs in an MR1-dependent manner[29]. However, when mice were treated with IFNAR blocking antibody then treated with 5-A-RU + MG, the state of DC maturation was largely unaffected (except for minor decreases in CD40). Therefore, there is evidence for type I IFN signalling in this model; however, it appears dispensable in the maturation of DCs.

One of the key pro-inflammatory cytokines involved in priming T cell responses is bioactive IL-12p70, constituting a heterodimer complex of IL-12p40 and IL-12p35. Analysis of serum cytokines after i.v. injection with 5-A-RU + MG showed no signs of circulating IL-12p40 or IL-12p70. This result is perhaps unsurprising since activated DCs could only be found within the lungs in this model which may constrain the amounts of IL-12 that make it into the systemic circulation. Therefore, it was important to assess direct production of IL-12 by the DCs themselves; however, intracellular staining for IL-12 is notoriously difficult. The use of IL-12p40^{eYFP} reporter mice revealed that indeed IL-12p40 production could only been seen in DCs within the lung. However, production of IL-12p40 is not directly indicative of the formation of IL-12p70 as it must combine with IL-12p35 to form bioactive IL-12. In fact, evidence in the literature suggests that homodimers of IL-12p40 have an inhibitory role, suppressing Th1 and Th17 responses through competitive binding of the IL-12 receptor[304–306], which may explain the lack of T cell expansion despite the presence of IL-12p40 producing DCs. Moreover, several studies have detailed the use of IL-12p40 homodimers as inhibitors of mouse models of inflammation[307,308]. Therefore, without direct measurement of IL-12p35 production in

DCs as a response to MAIT cell activation *in vivo*, it cannot be ruled out that MAIT cell-DC interactions result in the generation of inhibitory homodimers of IL-12p40.

It is also important to note that MAIT cells have been shown to induce IL-12p70 by DCs only when 5-OP-RU was combined with TLR agonists. When DCs were loaded with 5-OP-RU and then co-cultured with human MAIT cells, significant levels of IL-12p40 could be detected but not IL-12p70. However, when the TLR4 agonist, lipopolysaccharide (LPS), or the TL7/8 agonist, resiquimod (R848), were added to these co-cultures, the DCs were able to produce significant amounts of IL-12p70 in an MR1-dependent manner. This data demonstrates that MAIT cells activated by 5-OP-RU on DCs alone are unable to produce IL-12p35 to form bioactive IL-12p70. This may explain the inability of 5-A-RU + MG to adjuvant $CD8^+$ T cell responses when combined with OVA *in vivo*, as IL-12p70 is a critical cytokine in $CD8^+$ T cell priming. This is an interesting finding as NKT cells can induce IL-12p70 production by α-GalCer stimulation alone without TLR signalling. Perhaps one of the discrepancies between these two cell types is the ability to produce IFNγ in response to TCR stimulation, a cytokine which is known to regulate the expression of IL-12p35 specifically. At steady-state, the majority of NKT cells in the B6 mouse are NKT1 cells expressing T-bet and high amounts of IFNγ. In contrast, the majority of MAIT cells are MAIT17, expressing high levels of RORγt and IL-17. Thus, the over-representation of the MAIT17 subtype in B6 mice may result in failure to produce sufficient IFNγ in order to induce IL-12p35 in DCs. This further emphasizes the need to experimentally determine whether after injection with 5-A-RU + MG if lung resident DCs produce IL-12p35 as it may be a limiting factor in the formation of IL-12p70 to prime antigen-specific $CD8^+$ T cells.

To assess whether TLR stimulation would enhance MAIT cell-mediated DC functionality similar to the described *in vitro* studies above, 5-A-RU + MG was administered in combination with the TLR2 agonist, Pam2Cys. This combinations strategy resulted in enhanced maturation of DCs, which could not be achieved with either stimulus (at the doses used) alone. Importantly, when these stimuli were administered with OVA, expanded populations of endogenous OVA-specific $CD8^+$ T cells were observed in both the liver and the lung, in an MR1-dependent manner. Therefore, while the action of MAIT cells alone is insufficient to drive an appropriate level of maturation of the DCs, additional stimuli can overcome this deficit. It is assumed that this increased activity is through the

action of the TLR agonist on DC, although it is also possible that TLR stimulation directly augments MAIT cell functionality through TLRs on the cells themselves, perhaps through promoting the enhanced expression of CD40L or release of cytokines. Indeed, activated $CD4^+$ T cells can express TLR2 and produce higher levels of IFNγ when stimulated by bacterial lipopeptide[309]. Furthermore, it possible that TLR stimulation is enhancing T-bet expression in MAIT cells, resulting in increased IFNγ production. A recently published study modelling pulmonary infection with *Legionella longbeachae* shows that MAIT cells activated by bacterial infection but not 5-OP-RU alone were able to stimulate T-bet expression in MAIT cells[221]. This would suggest that in this model, TLR stimulation in combination with 5-A-RU + MG may be modulating MAIT cells towards a more Th1 and IFNγ producing phenotype which would favour $CD8^+$ T cell priming through enhanced IL-12p70 production. It has also been shown that TLR stimulation induces MR1 presentation by APCs *in vitro*, which in turn enhances MAIT cell activation[238,245]. A combination of these activities is entirely possible. At the doses used, TLR stimulation with Pam2Cys alone induced only a limited level DC maturation. When combined with MAIT cell activation, this MyD88-dependent expression of transcription factors such as NFκB[100,310] may co-operate with as yet undefined MAIT cell-mediated signals to DC to enhance activation status. Of relevance here, it has been shown that lung MAIT cells can be induced to proliferate after intranasally administered 5-OP-RU, but this requires co-administration of the TLR agonists Pam2Cys, CpG, or Poly I:C[246]. Like the observations here, in this model signals mediated via TLRs may be altering MAIT cell functionality directly, or through enhancing the capacity of APCs to stimulate MAIT cell activation and proliferation. This collective body of evidence highlights the importance of TLR co-operation in MAIT cell responses; however, the full extent and manner in which these co-operative activities affect adaptive immunity is not completely understood. The data here suggest signals mediated via TLR2 in particular can co-operate with MAIT cells to have impact on T cell responses. Whether other TLRs or indeed other forms of pattern recognition, can mediate similar cooperative activities remains to be established.

Similar co-operative properties have been shown between NKT cells and TLR ligands, where a combination of α-GalCer with a variety of different TLR ligands was shown to greatly enhance T cell responses to soluble antigens[21]. This was interpreted to reflect improved DC activity but, as above, the NKT cells could potentially respond to TLR stimulation directly via TLRs they express themselves. Indeed, it is known that NKT cells

express TLR4, and therefore can react directly to LPS. In experimental models of contact hypersensitivity, LPS has been shown to activate NKT cells in a TCR-independent manner to produce IL-4, which in turn activates B-1 cells to produce IgM and the recruitment of T cells[311–314]. Additionally, $CD1^{-/-}$ mice, which have no NKT cells, show defects in the ability to produce IFNγ, IL-12 and TNFα when administered LPS[314]. Together, these data suggest that NKT cells are major mediators in innate immune responses to TLR stimulation. It is possible that MAIT cells may be acting cooperatively with TLR stimulation in a similar manner.

In summary, this chapter details evidence for a novel role of MAIT cells within the lungs. Marked activation of lung resident DCs could be seen after i.v. injection with 5-A-RU + MG, which also resulted in the accumulation of activated DCs within the mLN. Activation of MAIT cells *in vivo* also resulted in the appearance of multiple myeloid populations within the lung tissue, revealing new roles for MAIT cells in modulating immune responses. MAIT cells alone were unable to provide sufficient help to drive adaptive $CD8^+$ T cell responses; however, in combination with TLR signalling could facilitate the expansion of antigen-specific $CD8^+$ T cells. There is still much investigation required in order to understand the exact mechanisms by which MAIT cells interact with DCs. However, this is the first evidence to support the concept that MAIT cells can act as cellular adjuvants, and provides a novel platform to explore the use of MAIT cell agonists in the design of novel immunotherapies.

Chapter 6: General discussion

6.1 Complications in the synthesis and use of 5-A-RU

Since the identification of the MAIT cell agonists, 5-OP-RU, 5-OE-RU and 5-MOP-RU and their precursor, 5-A-RU, numerous studies have detailed their use both *in vitro* and *in vivo* in order to investigate MR1 presentation and MAIT cell biology. Unfortunately, at the outset of this thesis efficient synthesis of the most potent known MAIT agonist, 5-OP-RU, was not yet achievable due to its unstable nature and proclivity to rapidly cyclise to inactive lumazines[210]. To circumvent these obstacles, the MAIT agonist precursor, 5-A-RU, which could be synthesised more readily, was used in this thesis to assess the influence MAIT cells have on adaptive immunity. Use of this precursor was initially regarded as an attractive approach, as 5-A-RU alone was also shown to be sufficient to drive MAIT cell activation due to availability of dicarbonyls as a byproduct of eukaryotic metabolism to condense with 5-A-RU to form MR1-antigens *in situ*. However, 5-A-RU also has stability issues, which were investigated in detail in this thesis.

6.1.1 Overcoming the instability of 5-A-RU

As had been noted earlier by other groups, 5-A-RU is prone to oxidation; however, the extent and consequences of this instability on MAIT cell activation had been understudied[231,236]. In one study, 5-A-RU was shown to degrade to unidentified products as early as 21 h when left at ambient temperature and exposed to light. However, the data in this thesis clearly shows that the propensity of 5-A-RU to undergo oxidative degradation was previously highly underestimated. In fact, loss of 5-A-RU occurs as early as a few minutes after exposure to oxygen in dilute aqueous solutions typically used in biological studies. Furthermore, incubation of these downstream oxidized products with human PBMCs revealed that they were unable to activate human MAIT cells. These findings are particularly relevant for immunologists who wish to use 5-A-RU, as this tendency to swiftly undergo oxidation occurs much faster than previously shown and could hamper reproducibility of biological studies. Thus, care should be taken when handling and storing 5-A-RU, and dilution to aqueous solutions should be minimized prior to use.

To circumvent these problems, 5-A-RU was conjugated to a cathepsin-cleavable VC-PAB linker to form a prodrug which greatly enhanced stability, even at room temperature. This stability was clearly demonstrated when the compound was exposed to air in

aqueous solution for up to 21 days without any signs of degradation. Stability of 5-A-RU has previously been achieved through preparation as an HCl salt which significantly improved shelf life[231]. However, the prodrug formulation used in this thesis has a significant additional advantage in that it was able to induce increased MAIT cell activation compared to native 5-A-RU. This was evident when 5-A-RU^{L} (compound 10) was compared to 5-A-RU in *in vitro* cultures with human MAIT cells, and when the activation of lung-resident MAIT cells was evaluated after intravenous administration into mice. It is possible that this increased MAIT cell activity could be attributed to the increased stability of 5-A-RU^{L}, and hence prolonged availability of the agonist precursor (after enzymatic release). The increased stability of the prodrug could be due to the electron-withdrawing properties of the VC-PAB linker, with the prodrug form preventing nucleophilic attack by chemical species such as oxygen. The linker may, therefore, confer protection of 5-A-RU en route to APCs in cell culture, or within systemic circulation after intravenous administration, where it may otherwise be susceptible to reactive species. This protection would, therefore, lead to an increase in intracellular concentrations of 5-A-RU which confers the enhanced MAIT cell activatory properties of 5-A-RU^{L}.

With the rise of research on MAIT cells, the synthesis and use of activatory compounds such as the MR1 ligand precursor 5-A-RU are becoming more widespread; however, not all laboratories have access to chemists able to produce such compounds. Therefore, with access being a limiting factor, a prodrug that can be shipped at room temperature without compromising integrity is a welcome advance to the field. However, despite the increased activity of the prodrug over native 5-A-RU, it was not as potent as 5-OP-RU, although the latter had to be prepared fresh for each experiment by combining 5-A-RU with MG. This, therefore, suggests that conversion of the prodrug to active agonist is not maximized, which could be due to either slow turnover into 5-A-RU or limiting MG. To overcome this disadvantage, a 5-OP-RU prodrug could be developed. Generation of a prodrug from 5-A-RU was achievable as the 5-amino group provided an accessible reactive site to attach the VC-PAB linker, and also conveniently impeded oxidative degradation, thereby enhancing stability. However, condensation of 5-A-RU with MG to form 5-OP-RU leads to loss of this amino group and consequently the original attachment site for the VC-PAB linker. To stabilise 5-OP-RU it would be necessary to impede its cyclisation into the weaker MR1 ligand, RL-7-Me. Furthermore, linkers that take advantage of release under acidic conditions such as within endosomes would be advantageous to ensure the

selective release of 5-OP-RU in proximity with MR1 molecules. The prospect of alternative linker chemistries that could fulfil these criteria is currently being explored through our collaboration with the Ferrier Research Institute.

6.1.2 A confounding NKT cell-activating factor in 5-A-RU and 5-A-RU conjugates

The assessment of 5-A-RU and peptide conjugate derivatives *in vivo* revealed a unique problem. While administration of 5-A-RU or 5-A-RU^{OVA} to mice induced activation of splenic DCs, and the conjugate induced peptide-specific responses, both of these activities were dependent on CD1d-restricted NKT cells which obscured the contribution of MAIT cells. It was initially thought to be possible that MAIT cells were somehow cross-activating NKT cells, which were then providing the majority of the DC stimulation. As similar findings were made in a collaborator's laboratory, where the source of 5-A-RU was different, we were confident in this initial hypothesis. However, fractionation and filtration of 5-A-RU in later experiments revealed that a microparticle contaminant was the cause of NKT-cell activation, which could be removed while retaining the MAIT cell-activating properties. It was presumed that a similar contaminating element was the cause of NKT cell activity within 5-A-RU^{OVA}; however, this could not be definitively assessed due to lack of sufficient material. A similar filtration method employed during the investigation of 5-A-RU may also prove difficult as 5-A-RU^{OVA} is non-polar and tend to form aggregates that could prevent filtration.

Unfortunately, the nature of this NKT cell-activating property could not be identified by the means explored in this thesis. As discussed, the possibility of 5-A-RU forming a CD1d ligand is likely to be low due to the physical constraints of the hydrophobic CD1d-binding pocket[267,268]. However, the possibility of this should still be explored by trying to form crystal structures of 5-A-RU with CD1d. An alternative explanation is that the manufacturing process for 5-A-RU itself introduced a contaminant that activates NKT cells directly or indirectly. A direct NKT cell-activating contaminant could be identified with the use of lipid digesting enzymes. A range of glycosphingolipids are known to dock CD1d and activate NKT cells[315]. As an example, with α-GalCer, the ceramide group binds to the CD1d groove through insertion of the phytosphingosine and acyl chain within the F' and A' pocket, respectively[267,316,317]. The enzyme imiglucerase, an analogue of the human β-glucocerebrosidase, cleaves sugars such as galactose from ceramides[318,319] that

are required to contact the NKT TCR and could be used to treat 5-A-RU preparations. Thus, elimination of NKT activity by imiglucerase would suggest a direct NKT cell-activating ligand contaminant. Alternatively, as discussed, an indirectly NKT cell-activating compound such as particulate matter could be activating DCs through an undefined mechanism. The chemical composition of the contaminants (i.e. inorganic or organic) could be analysed by a combination of microscopy methods such as TEM, SEM and EDS. It is important to reiterate that these NKT cell-activating properties were observed in 5-A-RU prepared from two separate laboratories, both of which manufacture lipid compounds. This would favour the former hypothesis that a direct NKT cell agonist is the contaminant. Moreover, in both labs, inconsistencies have been noted between batches of 5-A-RU in their ability to activate NKT cells and drive DC activation. These observations would, therefore, again favour the presence of a contaminant over an innate property of 5-A-RU to activate NKT cells.

The fact that an NKT cell activation was seen in two laboratories and could be filtered out is particularly relevant as the manufacture and use of 5-A-RU, and its derivatives are increasingly being used in the growing MAIT cell field. If the introduction of an NKT cell contaminating element can occur simply from the manufacturing process, this may be a systemic problem that is occurring in other laboratories that produce 5-A-RU. Therefore, it is possible that NKT cell activity could be contributing to the biological phenomenon observed when using 5-A-RU. It would, therefore, be of great interest to test other sources of 5-A-RU for NKT cell activity and whether this activity could be ameliorated by filtration.

New protocols have been established from the observations in this thesis in order to ensure that new 5-A-RU preparations are devoid of NKT cell activity. Using flow cytometry, lack of NKT activation can be confirmed by analysis of the spleen 18 hours after i.v. administration into mice. To eliminate any NKT activity, filtration of 5-A-RU stocks can be easily achieved by centrifugation through a 0.1μM filter. Subsequently, the ability of filtered 5-A-RU to activate MAIT cells can then be confirmed by testing a range of concentrations on human PBMCs *in vitro*.

6.2 Access of endosomal MR1 by a 5-A-RU prodrug

The canonical mode of presentation of antigen via MHC-I occurs firstly within the ER where appropriate binding of peptides ultimately results in translocation of MHC/peptide complexes to the cell surface for presentation towards T cells[38]. In addition to this, MHC-I molecules are continually internalised from the cell surface and are either targeted for destruction or may recycle to the cell surface after an exchange of antigen[320]. Recent studies have revealed that MR1 molecules follow similar pathways to MHC-I recycling where antigen can be captured in endosomes containing MR1 that has been recycled from the cell surface[205,213–215]. However, the biological implications of endosomal loading of MR1 versus the loading of MR1 within the ER in terms of downstream MAIT cell activation are currently unknown.

In this thesis, it was demonstrated that the 5-A-RU prodrug is almost exclusively loaded within recycling endosomes. Pre-saturating MR1 with Ac6-FP in THP cell lines revealed that 5-A-RU^{L} was loaded in a separate compartment to 5-A-RU. Inhibiting endosomal acidification with bafilomycin and linking MR1 molecules to the cell surface with GPI anchors then confirmed that 5-A-RU^{L} was being loaded within recycling endosomes (Fig 6.1). For MHC-I molecules, recycling from the cell surface represents an important mechanism to encounter antigens for cross-presentation[321,322]. In the case of MR1, accessing recycled MR1 molecules is likely a mechanism to directly sample microbial antigen within endocytic compartments. However, both soluble antigens such as 5-OP-RU and bacteria utilise ER-derived and recycled MR1 to present to MAIT cells. The necessity of 5-A-RU prodrug to load almost exclusively onto recycled MR1 molecules means it can be used to study the implications of endosomal loading of MR1 in isolation and eliminates confounding factors such as the pattern recognition likely to be triggered with whole bacteria that can potentially affect rate and level on MR1 presentation and induce co-stimulatory molecules[238,245]. It is also possible that access to recycled MR1 within endosomes confers the enhanced ability of 5-A-RU^{L} to activate MAIT cells when compared to 5-A-RU. It could be speculated that perhaps antigen presentation on MR1 within the recycling endosome results in altered spatiotemporal cell surface presentation in comparison to ER-derived MR1; although the mechanism in which this would occur is unknown.

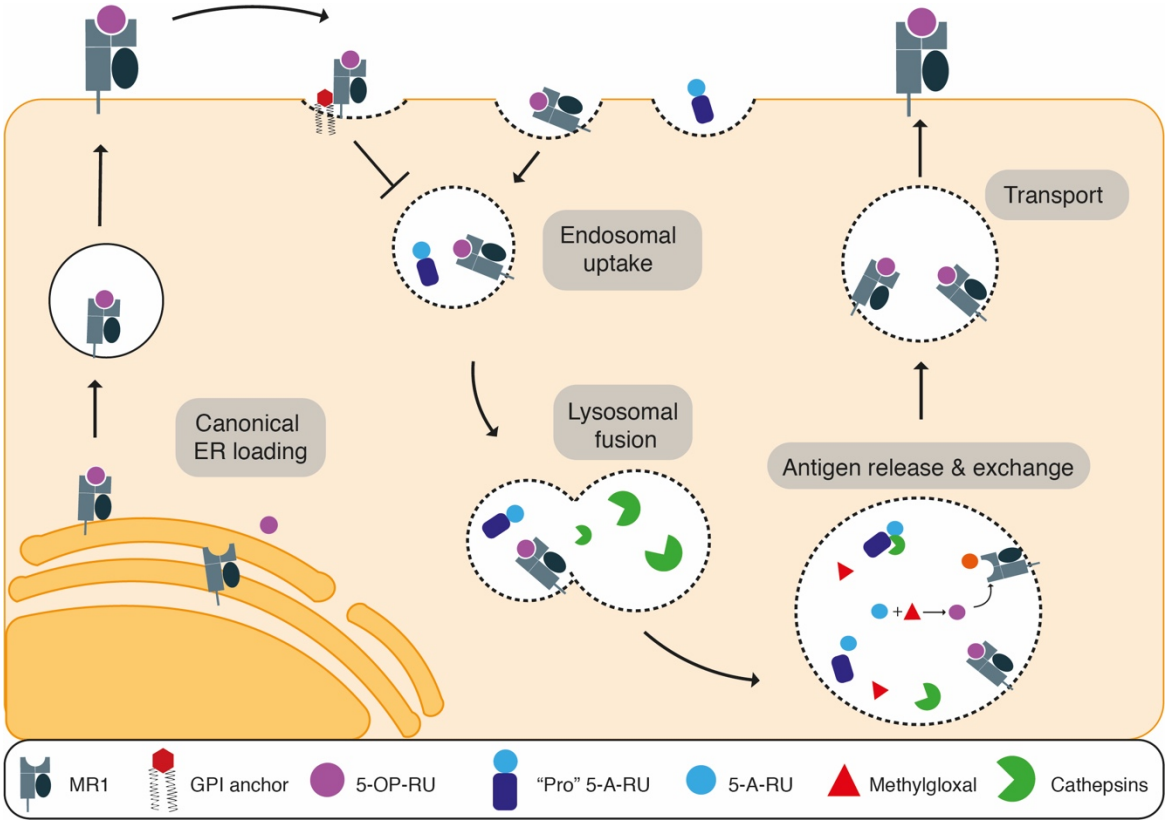


Figure 6.1 MR1 presentation of the 5-A-RU prodrug. Loading of endogenous soluble ligands leads to accumulation of MR1 on the cell surface which eventually leads to internalization into endosomes. Vesicles containing 5-A-RU^{L} fuses with incoming endosomes containing MR1. Subsequent lysosomal fusion of these late endosomes results in release of antigen from MR1. Endosomal cathepsins release 5-A-RU from the linker chemistry allowing condensation with MG to form 5-OP-RU. Antigen exchange can then occur onto MR1 allowing translocation and re-expression on the cell surface towards MAIT cells. Anchoring of MR1 molecules to the cell surface with GPI molecules inhibits uptake into endosomes and thus prevents presentation of 5-A-RU derived from the prodrug.

6.3 MAIT cells as cellular adjuvants to help induce T cell responses

Dendritic cells are a key subset involved in bridging the innate and adaptive immune responses. After the acquisition of antigen, the activation state of DCs is critically important to efficiently present these antigens in an immunogenic manner to naïve T and B cells[8–10]. Therefore, the study of adjuvants that can influence DC maturation is of high importance in the development of novel immunotherapies. After implementing strategies to eliminate NKT cell-activating contaminants, MR1-dependent and CD1d-independent DC activation within the lung could be achieved when 5-A-RU was first converted to 5-OP-RU by mixing with MG prior to i.v. administration to mice. However, priming of $CD8^+$ T cells could only be achieved when an antigen (in this case OVA) was administered with 5-A-RU + MG and the TLR2 agonist, Pam2Cys. Extra signals were therefore required for T cell priming, but at this stage, it remains unclear what these signals are.

An important observation made in previous *in vitro* studies was that 5-OP-RU could drive IL-12p40 production by DCs but required co-operation with TLR signalling to drive bioactive IL-12p70 expression[29]. The capacity to prime T cells *in vivo* when TLR stimulation accompanied MAIT cell activation may, therefore, be a result of enhanced IL-12p70 production in DCs; however, this was not directly investigated. It is interesting to speculate about other ways in which Pam2Cys may help T cell priming. It is possible that the signalling provided by MAIT cells to DCs via CD40L may not reach the required threshold in order for the DCs to prime T cells. Indeed, the level of co-stimulatory markers expressed on DCs by the administration of 5-A-RU + MG could be significantly increased by co-administering a low dose of Pam2Cys. Therefore, direct stimulation of MAIT cells by TLR agonists (via TLR on the MAIT cells) may improve their capacity to enhance co-stimulation via CD40L or production of IFNγ to enhance IL-12p35 production by DCs. The possibility of TLR signalling occurring on both MAIT cells and DCs should be investigated in future. A conditional knockout system such as *Itgax*-cre *Myd88*-flox that have the TLR adapter protein MyD88 knocked out in CD11c-expressing cells could be used to determine the contribution of TLR stimulation, such as with Pam2Cys, on DCs engaged in the priming of $CD8^+$ T cells. Conversely, *Cd3e*-cre *Myd88*-flox, where MyD88 is knocked out in CD3e expressing cells, may be a useful tool to

determine the role of direct TLR signalling on MAIT cells; however, this method may be confounded by TLR signalling acting on naïve $CD8^+$ T cells themselves[323]. The choice of TLR ligand should also be considered in future experiments. In this thesis, only Pam2Cys was explored. However, other TLR ligands have been described to enhance MAIT cell responses such as CpG and Poly I:C[246] which should be compared for their ability to enhance MR1-dependent DCs responses. Further, to explore whether MAIT cells fail to provide sufficient CD40L to DCs could be investigated in future experiments by co-administration of agonistic CD40.

The data from this thesis has presented clear new evidence that MAIT cells are able to influence the maturation state of DCs *in* vivo and when in combination with TLR agonists can drive antigen-specific $CD8^+$ T cell responses (Fig 6.2). *In vivo* research to date has shown that MAIT cells contribute to clearance of bacterial infection[187,247,324], which was thought to be mainly attributed to their ability to lyse infected cells[216,217]. This thesis provides another mechanism where bacteria provide both 5-OP-RU and TLR signalling to initiate MAIT-DC cross talk. This immunogenic interaction would enhance the presentation of bacterial derived antigens to generate antigen-specific T cell responses, therefore providing another mechanism MAIT cells may confer protection. A weakness in this thesis is that only the ability for MAIT cells to augment $CD8^+$ T cells was explored and not $CD4^+$ T cell responses. Future experiments should aim to determine whether MAIT cells activated by 5-A-RU + MG are sufficient to drive $CD4^+$ T cells responses or differentiation of $CD4^+$ T cells to T_{FH} cells. Priming of antigen-specific $CD8^+$ T cells when 5-A-RU + MG is co-administered with Pam2Cys may be in part due to an enhanced $CD4^+$ T cell responses. Further, an enhanced $CD4^+$ T_{FH} response may contribute to the development of B cell responses which should be investigated in future. This possibility could be investigated by CD4 depletion by antibody or I-A$\alpha^{-/-}$ mice (MHC-II deficient).

The expansion of antigen-specific $CD8^+$ T cells could be identified when 5-A-RU + MG was co-administered with Pam2Cys and OVA; however, their functionalities were not explored. Subcutaneous tumour models could be employed to characterise the ability of these $CD8^+$ T cells to infiltrate and lyse their target cells. Their ability to produce pro-inflammatory cytokines and express chemokine receptors should be measured and compared to other adjuvants for other innate-like T cells, such as α-GalCer, to determine whether targeting MAIT cells confer any significant advantage. More clinically relevant

antigens should also be used to examine further the ability of MAIT cells to act as adjuvants. The use of the HPV antigens E6/E7 could be explored here as pre-existing mucosal orthotopic models of the TC-1 cell line are available[325]. With these models, the use of MAIT cells as mucosal vaccines could be investigated. Further, targeting MAIT cells to stimulate responses against human self-antigens such as tyrosinase-related protein 2 (TRP-2), melanA and glycoprotein 100 (gp100)[326–330] should be investigated. Such antigens which are involved in differentiation of melanocytes are targets for immunotherapies for melanoma; however, are subject to tolerance mechanisms and require auxiliary signals such as adjuvants in order to prime $CD8^+$ T cells towards them. Thus, assessing the ability of MAIT cells to adjuvant such antigens would be informative in the translation of MAIT cell-based therapies into clinical studies. As previously described in studies utilising α-GalCer as an adjuvant, human PBMCs cultures could be employed to study whether MAIT cells can adjuvant T cells responses in human cells which can be tracked using pentamers that target specific TCRs[243].

Collectively, the data in this thesis suggests that MAIT cells could be harnessed therapeutically in order to generate such antigen-specific responses. It is well established that NKT cells are able to adjuvant potent antigen-specific adaptive responses *in vivo*[18–20,178–181], however, there is limited evidence for their ability to provide the same efficacy in the clinic which may in part be due to their decreased frequency in humans[22,182]. As MAIT cells are highly enriched within humans, up to 10% of circulating T cells, the data in this thesis suggest that MAIT cells could be a suitable candidate for vaccine development for use in the clinic.

Development of mucosal vaccines is of particular interest due to the high numbers of MAIT cells at mucosal tissues such as the lung. It has been shown in numerous studies that the mucosal based vaccinations are favourable in the generation of protective immunity against mucosal pathogens such as HIV, herpes virus, influenza virus and *Mycobacterium tuberculosis*[331–334]. In the case of HIV, the virus infects primarily through genito-urinary or genito-rectal mucosal surfaces[335]. Non-progression in patients with HIV is highly correlated with HIV-specific $CD8^+$ T cells[252]. However, previous vaccine strategies that utilised systemic delivery of vaccines failed to elicit HIV-specific immunity at mucosal surfaces which is thought to contribute to failure in protection[336]. Thus, the favourable positioning of MAIT cells at these mucosal surfaces provides an

opportunity to investigate whether they can be targeted in mucosal vaccine strategies as adjuvants. Such targeting of MAIT cells via mucosal vaccination could also prove beneficial for mucosal tumours such as in head and neck, lung and genital cancers. In a orthotopic murine model of head and neck cancer, it was shown that intranasal vaccination was far more efficient than intramuscular or subcutaneous administration at inducing effective antigen-specific $CD8^+$ T cell. Moreover, this route of immunization specifically stimulated mucosal integrins CD103 and CD49a on $CD8^+$ T cells which conferred enhanced tumour infiltration[337]. Similar benefits have been described in gastric, colorectal and bladder models of cancer where mucosal based vaccination strategies showed improved anti-tumour responses compared to subcutaneous administration[337–340]. Therefore, in future, it may be of interest to compare the functionality of antigen-specific T cells derived from MAIT cell-based vaccine strategies and whether they better mucosal homing properties. Moreover, alternative routes of administration should be explored to assess whether MAIT cells can activate DCs at distal mucosal sites.

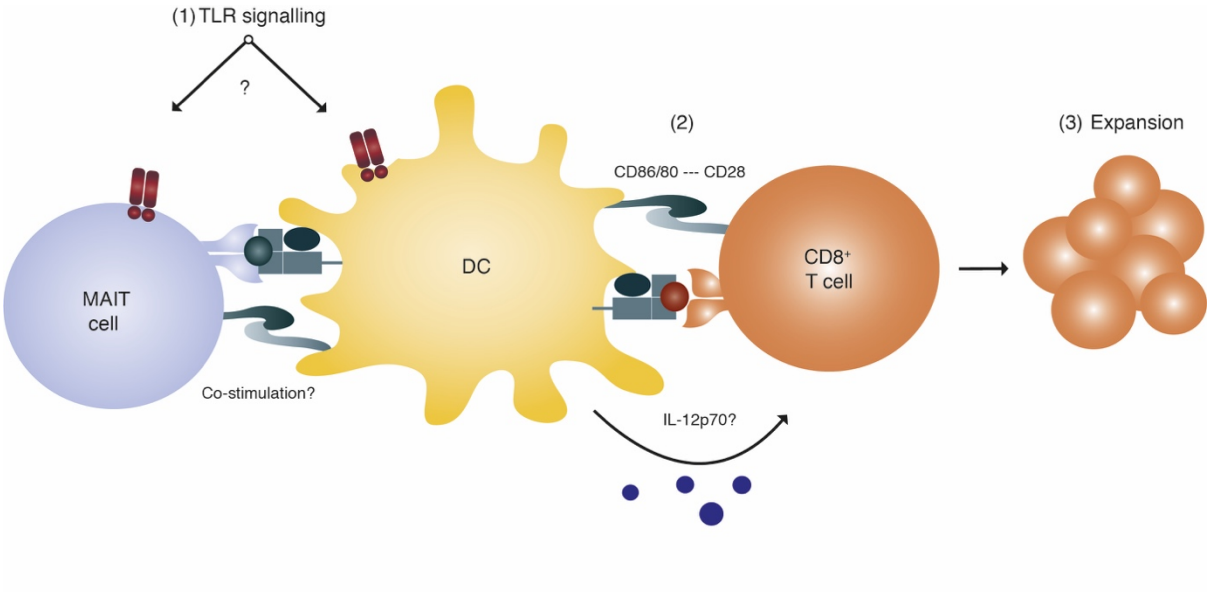


Figure 6.2 MAIT cells enhance DC functionality in order to prime CD8+ T cells. MAIT cells are activated by antigen loaded MR1 molecules on the DC. (1) TLR signalling on MAIT cells or DCs, or both, results in enhanced activation of the DC. (2) Consequently, DCs upregulate co-stimulatory markers such as CD86/80 that interact with naïve $CD8^+$ T cells, (3) ultimately leading to their proliferation.

.

6.4 A role for MAIT cells in Th2 immunity?

The study of MAIT cells in Th2 responses is limited, primarily due to the lack of reported GATA3 expression in MAIT cells and the fact that they do not readily produce Th2 cytokines such as IL-4 and IL-13. In addition to the findings that MAIT cells were influencing DC maturation in the lung, flow cytometric analysis of other cell populations as well as analysis of serum cytokines after injection with 5-A-RU + MG revealed MAIT cells were also influencing other immunological events. An MR1-dependent increase of the Th2 cytokine, IL-5, and G-CSF could be seen in the serum of treated mice. Moreover, a marked increase in eosinophils and neutrophils, known to be associated with Th2 responses, could be seen in the lungs.

Given the infiltration of eosinophils into the lungs seen in this model, it's interesting to consider the possible role that MAIT cells may play in the inflammatory response to helminth infections which are known to provoke strong Th2 responses including eosinophilia. Helminths feed on bacteria, and some studies have reported helminths may act as vectors for pathogenic strains of bacteria which survive internally within the helminth[341–343]. Some strains found in these studies include bacteria that are known to express the riboflavin operon, which can result in the formation of MAIT antigens. It's intriguing to postulate that during helminth infection, MAIT cells may be reacting to these antigens and support the recruitment and activation of eosinophils. It is unclear whether an increase in eosinophilia within the lung after MAIT cell activation would confer protection against helminth infections due to conflicting evidence in the literature (reviewed here[344]). The increase of neutrophils would also suggest further roles of MAIT cells in Th2 immunity as neutrophils are heavily implicated in helminth infections where they are rapidly recruited, activated and mediate protection as well as tissue remodelling[345–347].

Inappropriate activation and recruitment of eosinophils is also a hallmark of asthma[348]. An increase in circulating IL-5 and eosinophils into the lung suggests that MAIT cells may have a role in the pathogenesis of asthma, although the functionality of these eosinophils is unknown in this model. Clear evidence for a role of MAIT cells in asthma is yet to be established. In one study, increased levels of IFNγ-producing MAIT cells in the blood at 1 year of age correlated with decreased risk of asthma at age 7[349]. In another

study circulating IL-17 producing MAIT cells were associated with increased asthma severity in children[350]. Together these studies would suggest that the MAIT1 subset provides a protective role while MAIT17 is associated with pathology; however, no such experimental data exists to substantiate a causal link. Moreover, there is no evidence yet that the association between MAIT cells and asthma could be attributed by an ability to influence Th2 immune responses.

It is unclear from the work in this thesis whether MAIT cells are directly exhibiting Th2 cell properties or if their activation results in cross-activation and recruitment of innate cells such as eosinophils and neutrophils. The contributions of MAIT cells in infection models that evoke Th2 immune responses could be explored with $MR1^{-/-}$ mice. The nematode *Nippostrongylus brasiliensis* infects rodents and enters the lungs during their L3 developmental stage where they induce strong eosinophilia[351]. Thus, in this model, the role of MAIT cells in protection against *N. brasiliensis* could be determined by enumerating the number of eosinophils or live worms in the lung when infected in $MR1^{-/-}$ compared to WT.

Fluorescent cytokine reporter mice could be used to explore the possibility that MAIT cells are directly producing Th2 cytokines. The IL-5 tdTomato[352] or the 4C13R[353] reporter mouse could be used to assess if MAIT cells are expressing IL-5 or IL-4 and IL-13, respectively. Moreover, a larger-scaled cytometric analysis including a wide array of markers could be conducted in order to identify as many cell types as possible, including activation markers or other markers of cellular function such as the release of cytokines. During Th2 inflammation an array of immune cell types may be involved such as mast cells, granulocytes and innate lymphoid cells which may be contributing to observations made here. Technologies such as high dimensional spectral flow cytometry, or mass cytometry (CyTOF), would be useful tools in this analysis, as upwards of 35 markers can be assessed at any one time in a sample.

6.5 Conclusion

In conclusion, this thesis has shown that the synthetic MAIT cell agonist precursor 5-A-RU, like the agonist 5-OP-RU itself, is unstable. The precursor is very sensitive to oxidation and rapidly forms products that fail to form agonists that can activate MAIT cells. Modification of 5-A-RU by chemical conjugation of a cathepsin-labile linker to form a prodrug enhanced stability, conferred enhanced MAIT cell activation, and enabled attachment of peptide antigens (and other moieties of choice). Unlike unmodified 5-A-RU, the agonists formed from the prodrug were loaded onto MR1 primarily in recycling endosomes rather than the ER. Despite these attributes, the prodrug was unable to achieve the level of MAIT cell activity induced by admixing 5-A-RU with MG to form 5-OP-RU before use, although this latter process was hard to control. Although manufacturing issues that lead to NKT cell activation confounded initial *in vivo* investigations with MAIT cell agonists in this thesis, later studies showed that administration of 5-OP-RU could activate DC in the lungs. However, co-administration of soluble antigen with 5-OP-RU was insufficient to drive antigen-specific $CD8^+$ T cell responses, suggesting further signals to the DC were required. Indeed, when antigen and 5-OP-RU were administered in concert with TLR2 stimulation, antigen-specific $CD8^+$ T cell responses were induced. Moreover, activation of MAIT cells resulted in the appearance of additional immune subsets in the lung, including eosinophils, macrophages and neutrophils, which point towards a broader role for MAIT cells in inflammatory disease settings. Collectively, the data in this thesis detail a role for MAIT cells in influencing innate and adaptive immunity, and suggest that, with the supply of appropriate signals, there may be a therapeutic potential for these cells in future vaccine design.

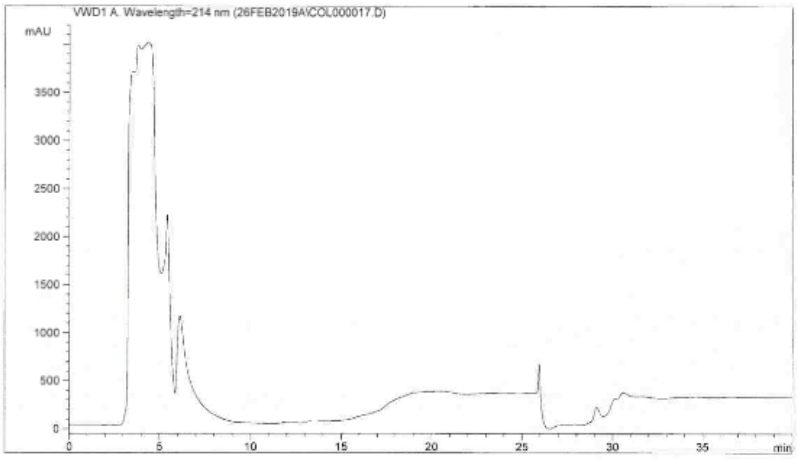
Appendices

Appendix A

Data File D:\CHEM32\1\DATA\2019\FEB19\26FEB2019A\COL000017.D

```
Acq. Operator   :
Acq. Instrument : Instrument 1                    Location : Vial 1
Injection Date  : 3/6/2019 4:53:34 PM
Acq. Method     : C:\CHEM32\1\METHODS\PREP10137A.M
Last changed    : 3/6/2019 4:18:44 PM
                  (modified after loading)
Analysis Method : C:\CHEM32\1\METHODS\PREP10137A.M
Last changed    : 3/6/2019 6:34:12 PM
                  (modified after loading)
Method Info     : Purification of Regan's Sample
                  Column : Phenomenex Gemini C18 110A 250 x 10 mm 5 um
                  Guard : Phenomenex SecurityGuard 10 x 10 mm Gemini C18
                  Mobile Phase A: 95:5:0.05  Water : Methanol : TFA
                  Mobile Phase B: 100:0.05  Methanol :TFA
                  Mobile Phase C: ( using A ) 90:10:0.05 Methanol : IPA : TFA
                  Gradient (A:B) : T0=100:0, T5=100:0, T15=0:100, T20=0:100,
                  (Switch A to MP C:), T20.5=100:0, T40=100:0. (Switch A to MP A)
                  Sample Solvent :  ? , Flow Rate = 5 ml/min
                  Detection : 214 nm,    Column Temp 20 C (initial) then 40 C

Sample Info     : ~~blank inj~~ (0.5 mL water) 104 bar
                  Sample run (500µL)
```




```
                          Area Percent Report

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs

No peaks found

                          *** End of Report ***
```

Instrument 1 3/6/2019 6:34:16 PM Page 1 of 1

Figure A. Isolation of 5-A-RU by HPLC. Isolation was performed using a Phenomenex Gemini C18 reverse-phased column. A 500µl sample 5-A-RU at 13mgmL^{-1} was loaded onto the column for isolation. Elution was performed by the addition of a mobile phase to the column as follows: 0 – 5 min, 95% water + 5% methanol, 5 – 15 min, 95% water + 5% - 100% methanol (linear gradient), 15 – 20 min, 100% methanol, 20 – 40 min, 90% methanol + 10% isopropanol. All solvents contained 0.05% trifluoroacetic acid. The solvents were applied to the column at a flow rate of 5mL/min. UltraViolet light at 214nm was utilised to identify compounds by spectroscopy. Fraction 1 containing 5-A-RU was detected and eluted between 2 min – 7 min 40 s. Performed by Regan J Anderson.

Appendix B

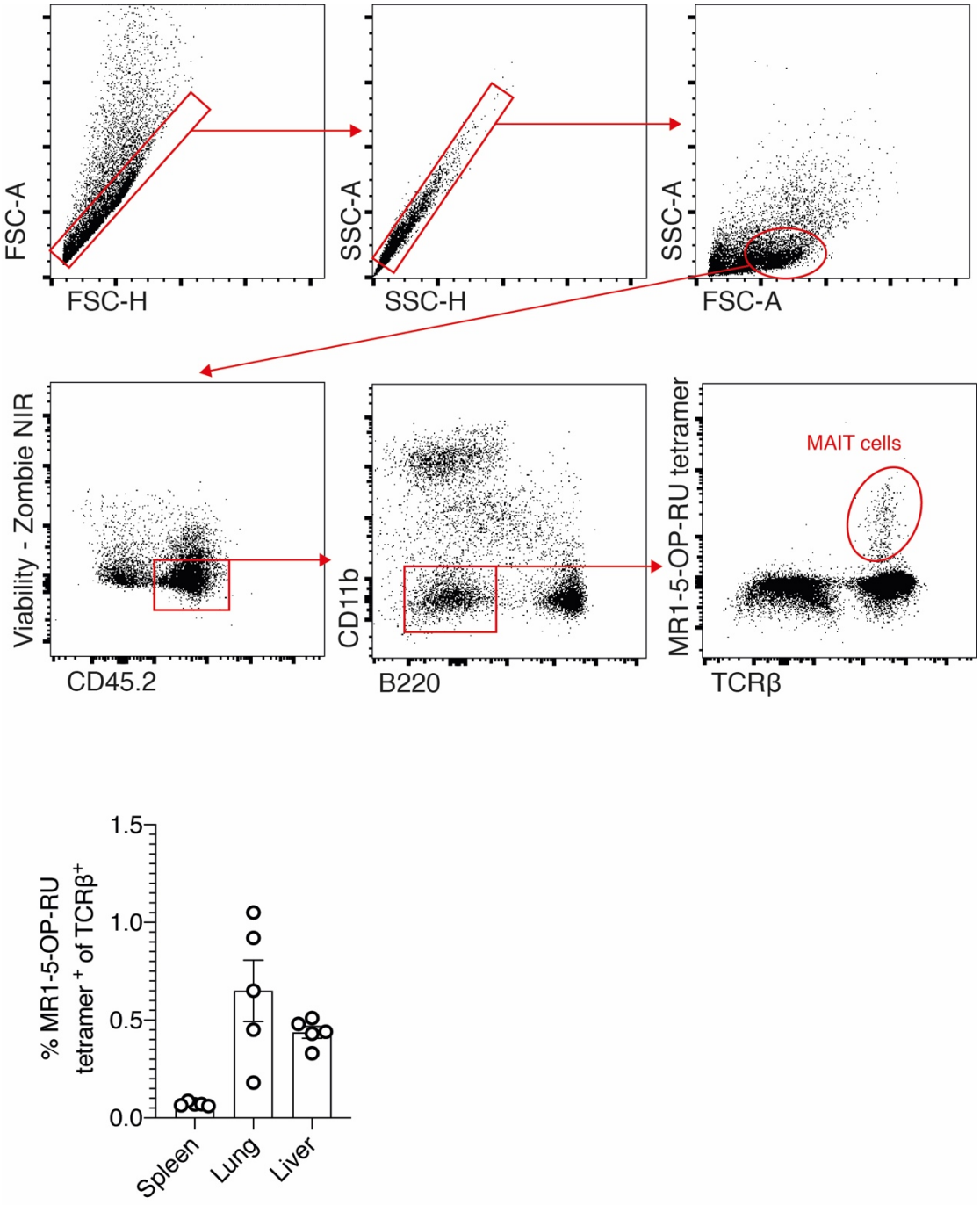


Figure B. *In vivo* gating strategy and tissue distribution of MAIT cells. Representative flow cytometry gating strategy for MAIT cells in the lungs of mice. When analysing the liver and the spleen, the same gating strategy was used and yielded similar results. Graph shows frequency of MAIT cells across the specified tissues where MAIT cells are gated as shown above.

Appendix C

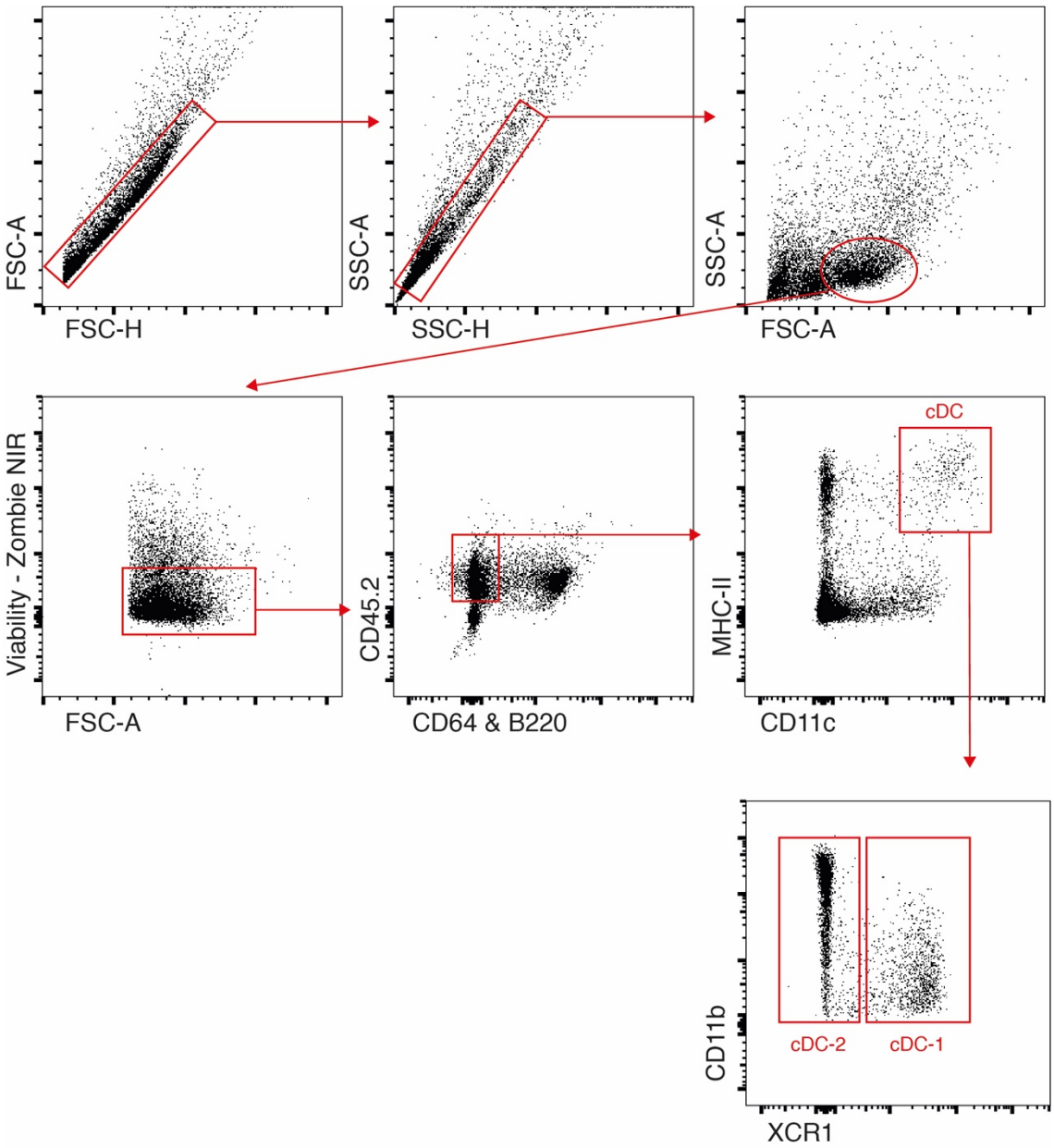


Figure C. *In vivo* gating strategy for cDCs. Representative flow cytometry gating strategy for cDCs in the lungs of mice. When analysing the liver and the spleen, the same gating strategy was used and yielded similar results.

Appendix D

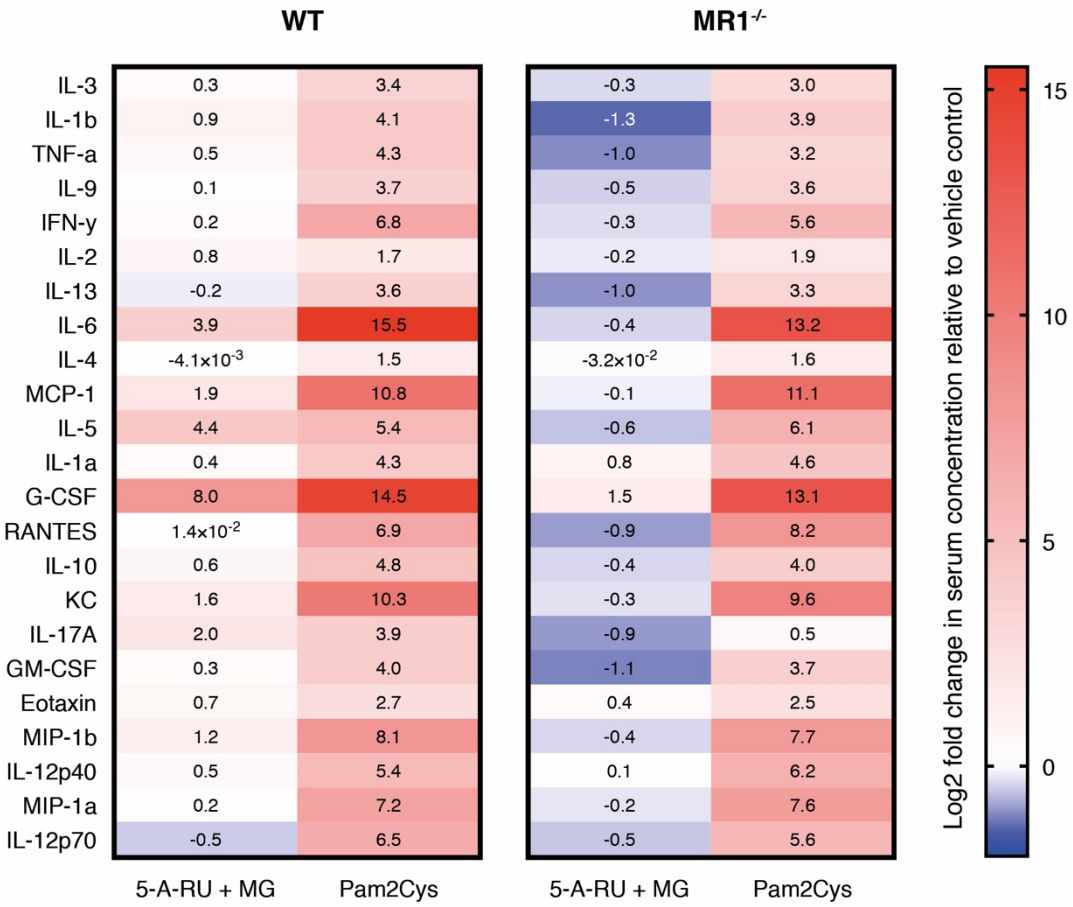


Figure D. Serum cytokines 6 h post i.v. WT or MR1$^{-/--}$ mice (n=5) were injected with 5-A-RU + MG or Pam2Cys i.v. and bled after 6 h for serum. Serum was analysed by bioplex to assess cytokine production. Data representative from a single experiment. Numbers represent the fold change relative to control for each cytokine after normalization of the mean concentrations to that of the control group and subsequent Log_2 transformation.

Appendix E – Patient consent and information for blood donation

Frequency and function of innate-like T cells in patients with cancer

Principal Investigator: Dr Robert Weinkove. (Robert.Weinkove@ccdhb.org.nz /04 385 5999 via hospital switchboard)

CONSENT FORM FOR PARTICIPANTS

Following signature and return to the research team this form will be stored in a secure place for ten years.

Name of participant:……..

1. I have read the Information Sheet for this study and understand the aims of this research project.
2. I have had enough time to talk with other people of my choice about participating in the study.
3. I confirm that I meet the criteria for participation which are explained in the Information Sheet.
4. All my questions about the project have been answered to my satisfaction, and I understand that I am free to request more information at any stage.
5. I know that my participation in the project is entirely voluntary, and that I am free to withdraw from the project at any time without disadvantage.
6. I know that as a participant I will undergo a blood test as part of this study, giving approximately 25ml of blood.
7. I understand that non-identifiable information may be shared with other researchers for future analyses of innate-like T cells.
8. I know that the questionnaire will ask some health questions. If I feel hesitant or uncomfortable I may decline to answer any particular question(s), and /or may withdraw from the project without disadvantage of any kind.
9. I agree to the investigators reviewing my medical records in order to link the stage, type and outcome of any malignancy with innate-like T cell numbers and function. I understand this information will be kept confidential.
10. I understand the nature and size of the risks of discomfort or harm which are explained in the Information Sheet.

11. I know that when the project is completed all personal identifying information will be removed from the paper records and electronic files which represent the data from the project, and that these will be securely stured and kept for ten years.
12. I understand that results of this study may be published and available in the University of Otago Library and that I will not be identifiable in study reports.
13. I know that there is no remuneration offered for this study, and that no commercial use will be made of the data.
14. I understand that the blood samples will be transported to and stored securely at the Malaghan Institute of Medical Research, and that the researchers will dispose of my samples when they are no longer necessary for this research project, unless I have provided separate written consent for future research.
15. I would like a karakia prior to destruction of by blood cell samples: **Yes / No**
16. Optional: I agree to be contacted by the researchers to ask whether I would like to donate future blood samples for this study: **Yes / No**

Signature of participant:	Date:

Signature and name of witness (optional):	Date:

Optional
If you would like a summary of the results of this study, or if you agree to be contacted by the researchers to ask about donating future blood samples, please provide at least one contact method below.

Telephone: _____

OR Email: _____

OR Address: _____

_____

Future Unspecified Research – Innate-like T cells and cancer

Principal Investigator: Dr Robert Weinkove. (Robert.Weinkove@ccdhb.org.nz /04 385 5999 via hospital switchboard)

INFORMATION SHEET FOR PARTICIPANTS

What is future unspecified research?

You have been asked to take part in a study of innate-like T cells in patients with cancer and in healthy volunteers. That study involves donating blood to allow for laboratory investigation of immune cell number and function. Normally, all of the cells, whether used for the research or not, would be destroyed at the end of the above research project.

However, it is possible that leftover cells that you have donated may be useful for future research projects ('future unspecified research'). Therefore, we are asking whether you would like to donate any remaining cells to the Malaghan Institute of Medical Research for this purpose.

This information sheet explains why we are asking you to donate remaining cells for future research, and what would happen if you choose to participate. Agreeing to donate remaining cells is voluntary – you do not have to agree to this in order to take part in the main study.

As with the main study, this requires the storage of leftover blood products (human tissue). We acknowledge that there may be cultural issues with storing tissue for extended periods of time. As such, we encourage you to include your family/whānau in discussions for all stages of the research.

What will happen to my cells if I agree to this, and how will they be disposed of?

As a of the main study, your blood cells will already be cryopreserved (frozen) at the Malaghan Institute of Medical Research in Wellington. If you were to agree to this, the blood cells will be stored indefinitely in a very low temperature freezer at the Malaghan Institute of Medical Research, even after the current research project ends. At some time in the future your blood cells may be thawed and studied for future research projects a the Malaghan Institute. If you specifically agree to this in the consent form, your cells may be sent to other New Zealand researchers. Your blood cells will be identified only by a code number that the researchers will be able to decode.

If you donate your remaining cells for future research, they will be destroyed at the earliest of the following: when there are insufficient cells left for future research, when the researchers think that your cells are not likely to be useful for future research, when there is no longer enough storage capacity to keep your cells, OR if you ask for them to be destroyed. If you have indicated that you would like a karakia performed prior to cell disposal on the consent form for the main study, this will be honoured at this point. Remaining cells are safely disposed of via incineration. Due to the addition of chemicals for storage and analysis, we are unable to return cells to you.

What research might be done with my cells?

There are many different ways in which the cells could be used and we cannot be specific about what tests might be done on them, but it is likely to be related to the further development of vaccines for the treatment of cancer and infectious diseases. Your cells will not be used for germline (hereditary) genetic analyses. Any research that is carried out on your blood cells will be approved by an accredited Ethics Committee, to ensure that it is in agreement with the original consent that you provided.

How will my information be used?

The results of any future unspecified research may be published in scientific journals and will therefore be made available to everyone. Even if your cells have been used, you will not be identified in any published material. Because information is freely shared by the international scientific community, and because sometimes collaborating with a commercial enterprise is the best way to progress a new technique, it is not possible to rule out the possibility that information derived from research on your blood cells will ultimately result in financial gain to someone.

Any questions?

If you have any questions now or in the future, please feel free to contact:

Dr Robert Weinkove Telephone: 04 385 5999 (via hospital switchboard)

This study has been approved by the University of Otago Human Ethics Committee (Health). If you have any concerns about the ethical conduct of the research you may contact the Committee through the Human Ethics Committee Administrator (phone +64 3 479 8256 or email gary.witte@otago.ac.nz). Any issues you raise will be treated in confidence and investigated and you will be informed of the outcome.

Research Advisory Group – Māori
Māori Partnership Board, Capital & Coast DHB

RESEARCH ADVISORY GROUP MĀORI (RAG-M)

25 November 2015

Malaghan Institute
PO Box 7060
Wellington 6242
Tēnā koe

RAG-M 2015/413

On behalf of the Research Advisory Group Māori I write in relation to your study titled: **Frequency and function of innate like T-cells in patients with cancer**

We have based our assessment of your research on the following documentation you have supplied:

- RAGM cover sheet
- Study protocol
- Patient information and consent forms
- Ethics application

We acknowledge the consideration that you have made for potential Māori participants and ensuring that cultural requirements are met, particularly regarding the handling of tissue (blood samples), and with the inclusion of whānau in the decision making regarding study participation.

In collecting ethnicity data, we request that you use the format of the New Zealand Census Question. Please clarify whether this format is used.

We acknowledge also that you have considered cultural issues surrounding tissue samples; however we have found it difficult to locate all the data regarding tissue within the patient information and consent form. The information is in various places in the form, and further information could be added. We would recommend including the following information in one section/paragraph:

- The number of samples to be taken
- The amount of blood to be taken
- How the sample is transported
- Where it will be analysed (ie; where the Malaghan institute is located)
- Method of destruction

Appendix F – Chemistry methodology

Methods for chemical analysis are from: The chemical synthesis, stability and activity of MAIT cell prodrug agonists that access MR1 recycling endosomes. (2020) ACS Chem. Bio. Doi: 10.1021/acschembio.9b00902.

LCMS

Method A: Analytical HPLC was performed on an Agilent 1260 HPLC with an Agilent 6130 single quadrupole mass spectroscopic detector using ESI. A Phenomenex Synergi^{TM} Fusion-RP C18 column (2.5 μM, 3x50 mm) was installed, heated to 35 °C, with 10 mM aq NH_4OAc (pH 6.0) used as eluent A and MeOH as eluant B (flow = 0.5 mL/min). The following gradient method was used: T0 (min) = 100:0 A:B, T1.5 = 100:0 A:B, T1.51 = 90:10 A:B, T3.5 = 70:30 A:B, T4 = 100:0 A:B, T7 = 100:0 A:B. Peaks were detected by UV absorbance, primarily at 254 nm, and identified with the inline MS detector.

Method B: As above in Method A with the following modifications: Eluent = 10 mM aq NH_4OAc (pH 6.3) (isocratic), 20 °C.

Method C: Analytical HPLC was performed on an Agilent 1260 HPLC with an Agilent 6130 single quadrupole mass spectroscopic detector using ESI. An Agilent Poroshell 120 EC-C18 column (2.7 μM, 3x50 mm) was installed, heated to 40 °C, with water (+0.1% formic acid) used as eluent A and MeOH as eluant B (flow = 0.5 mL/min). The following gradient method was used: T0 (min) = 70:30 A:B, T8 = 0:100 A:B, T9 = 0:100 A:B, T10 = 70:30 A:B, T12 = 70:30 A:B. Peaks were detected by UV absorbance, primarily at 254 nm, and identified with the inline MS detector.

Method D: A Phenomenex Synergi^{TM} Fusion-RP C18 column (2.5 μM, 3x50 mm) was installed, heated to 40 °C, with water (+0.1% formic acid) used as eluent A and MeOH as eluant B (flow = 0.5 mL/min). The following gradient method was used: T0 (min) = 100:0 A:B, T4 = 100:0 A:B, T7 = 70:30 A:B, T8 = 100:0 A:B, T10 = 100:0 A:B. Peaks were detected by UV absorbance, primarily at 254 nm, and identified with the inline MS detector.

Method E: A Phenomenex Kinetex C18 column (100 Å, 2.6 μM, 3x50 mm) was installed, heated to 40 °C, with water (+0.1% formic acid) used as eluent A and MeOH as eluant B (flow = 0.5 mL/min). The following gradient method was used: T0 (min) = 95:5 A:B, T8 = 0:100 A:B, T12 = 0:100 A:B, T13 = 95:5 A:B, T15 = 95:5 A:B. Detection was by selected ion monitoring (SIM) MS. The traces shown in Fig 8 of the main article represent the combined SIM chromatograms for prodrug substrate, expected cleavage product and internal standard (IS). Conversion was determined by comparing the ratio of substrate:IS at a given time point to the ratio at t=0.

5-A-RU degradation studies

For analysis of 5-A-RU prepared by hydrogenolysis of nitrouracil, an aliquot was removed by syringe and diluted 60-fold with deoxygenated water and analysed immediately by LCMS (Method A). A second aliquot was diluted similarly and the solution (30 μL) was gently agitated in air over 8 min. The solution was transferred to a capped HPLC vial and analysed by LCMS (Method A) at the timepoints indicated.

For analysis of 5-A-RU prepared by dithionite reduction of nitrouracil and aliquot of 5-A-RU was diluted 130-fold with deoxygenated water in an Ar-filled HPLC vial and analysed immediately by LCMS (Method B). A second aliquot was diluted similarly and the solution (240 μL) was gently agitated in air over 3 min. The solution was transferred to a capped HPLC vial and analysed by LCMS (Method B) at the timepoints indicated in Figure 3 of the main article.

Prodrug degradation studies

In aqueous media*:* Prodrug **10** was dissolved in 1:4 DMSO/water to 0.2 mg/mL. Carbamate **12** was dissolved in water to 0.2 mg/mL. 100 μL samples were shaken in uncapped vials on an automatic shaker for 24 h, then capped and stored at rt protected from light. Aliquots were analyzed by LCMS (Method C for **10**, Method D for **12**) at the timepoints indicated.

In DMSO*:* Prodrug **10** was dissolved in neat DMSO to 0.2 mg/mL, and stored either at rt protected from light, or at -20 °C. Aliquots were analyzed by LCMS (Method C) at the timepoints indicated.

Cathepsin B assay

Cathepsin B assays were conducted in NH_4OAc buffer: NH_4OAc (50 mM, pH 5.3) + EDTA (2.5 mM) + dithiothreitol (2.5 mM). Phytosphingosine was used as in internal standard for LCMS analysis. A stock solution of phytosphingosine in DMSO (190 μM) was pre-mixed with NH_4OAc buffer to a final phytosphingosine concentration of 6.3 μM. The prodrug substrate (190 μM in DMSO) was added to the pre-mixed buffer solution to give a final substrate concentration of 12.7 μM. Cathepsin B from human liver (Sigma) was diluted to 1 unit/mL in NH_4OAc buffer and added to the reaction mixture to give a final cathepsin B concentration of 0.27 units/mL. For the control reaction (without enzyme) the same volume of buffer was added. The reaction mixtures were then incubated at 37 °C. Aliquots of 10 μL were taken from the reactions and analysed by LCMS (Method E) at 30 min and 4 h after the reaction was initiated.

Publications

Publications arising from work presented in this thesis:

1. **J Lange**, RJ Anderson, AJ Marshall, STS Chan, TS Bilbrough, O Gasser, C Gonzalez-Lopez, M Salio, V Cerundolo, IF Hermans, GF Painter. *The chemical synthesis, stability and activity of MAIT cell prodrug agonists that access MR1 recycling endosomes.* (2020) ACS Chem. Bio. Doi: 10.1021/acschembio.9b00902

2. **J Lange,** K Buick, RJ Anderson, AJ Marshall, O Gasser, M Salio, L Connor, GF Painter, IF Hermans. *MAIT cells influence the activation of lung resident dendritic cells.* In preparation.

References

1. Trombetta, E. S. & Mellman, I. Cell Biology of Antigen Processing in Vitro and in Vivo. *Annu. Rev. Immunol.* **23**, 975–1028 (2005).
2. Akira, S., Uematsu, S. & Takeuchi, O. Pathogen recognition and innate immunity. *Cell* vol. 124 783–801 (2006).
3. Kawai, T. & Akira, S. The role of pattern-recognition receptors in innate immunity: Update on toll-like receptors. *Nature Immunology* vol. 11 373–384 (2010).
4. Arens, R. & Schoenberger, S. P. Plasticity in programming of effector and memory CD8+ T-cell formation. *Immunological Reviews* vol. 235 190–205 (2010).
5. Harrington, L. E., Janowski, K. M., Oliver, J. R., Zajac, A. J. & Weaver, C. T. Memory CD4 T cells emerge from effector T-cell progenitors. *Nature* **452**, 356–360 (2008).
6. Prlic, M., Williams, M. A. & Bevan, M. J. Requirements for CD8 T-cell priming, memory generation and maintenance. *Current Opinion in Immunology* vol. 19 315–319 (2007).
7. Hoffman, W., Lakkis, F. G. & Chalasani, G. B cells, antibodies, and more. *Clin. J. Am. Soc. Nephrol.* **11**, 137–154 (2016).
8. Hawiger, D., Inaba, K., Dorsett, Y., Guo, M., Mahnke, K., Rivera, M., Ravetch, J. V., Steinman, R. M. & Nussenzweig, M. C. Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. *J. Exp. Med.* **194**, 769–779 (2001).
9. Iberg, C. A., Jones, A. & Hawiger, D. Dendritic Cells As Inducers of Peripheral Tolerance. *Trends in Immunology* vol. 38 793–804 (2017).
10. Finkelman, F. D., Lees, A., Birnbaum, R., Gause, W. C. & Morris, S. C. Dendritic cells can present antigen in vivo in a tolerogenic or immunogenic fashion. *J. Immunol.* **157**, 1406–14 (1996).
11. Michelsen, K. S., Aicher, A., Mohaupt, M., Hartung, T., Dimmeler, S., Kirschning, C. J. & Schumann, R. R. The role of toll-like receptors (TLRs) in bacteria-induced maturation of murine dendritic cells (DCs): Peptidoglycan and lipoteichoic acid are inducers of DC maturation and require TLR2. *J. Biol. Chem.* **276**, 25680–25686 (2001).
12. Ahrends, T., Spanjaard, A., Pilzecker, B., Bąbała, N., Bovens, A., Xiao, Y., Jacobs, H. & Borst, J. CD4+ T Cell Help Confers a Cytotoxic T Cell Effector Program

Including Coinhibitory Receptor Downregulation and Increased Tissue Invasiveness. *Immunity* **47**, 848-861.e5 (2017).

13. Crotty, S. A brief history of T cell help to B cells. *Nat. Rev. Immunol.* **15**, 185–189 (2015).
14. Smith, C. M., Wilson, N. S., Waithman, J., Villadangos, J. A., Carbone, F. R., Heath, W. R. & Belz, G. T. Cognate CD4+ T cell licensing of dendritic cells in CD8+ T cell immunity. *Nat. Immunol.* **5**, 1143–1148 (2004).
15. Schoenberger, S. P., Toes, R. E. M., Van Dervoort, E. I. H., Offringa, R. & Melief, C. J. M. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD4OL interactions. *Nature* **393**, 480–483 (1998).
16. Cella, M., Scheidegger, D., Palmer-Lehmann, K., Lane, P., Lanzavecchia, A. & Alber, G. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *J. Exp. Med.* **184**, 747–752 (1996).
17. McAdam, A. J., Greenwald, R. J., Levin, M. A., Chernova, T., Malenkovich, N., Ling, V., Freeman, G. J. & Sharpe, A. H. Icos is critical for CD40-mediated antibody class switching. *Nature* **409**, 102–105 (2001).
18. Fujii, S. I., Liu, K., Smith, C., Bonito, A. J. & Steinman, R. M. The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. *J. Exp. Med.* **199**, 1607–1618 (2004).
19. Hermans, I. F., Silk, J. D., Gileadi, U., Salio, M., Mathew, B., Ritter, G., Schmidt, R., Harris, A. L., Old, L. & Cerundolo, V. NKT Cells Enhance CD4 + and CD8 + T Cell Responses to Soluble Antigen In Vivo through Direct Interaction with Dendritic Cells . *J. Immunol.* **171**, 5140–5147 (2003).
20. Stober, D., Jomantaitė, I., Schirmbeck, R. & Reimann, J. NKT Cells Provide Help for Dendritic Cell-Dependent Priming of MHC Class I-Restricted CD8 + T Cells In Vivo . *J. Immunol.* **170**, 2540–2548 (2003).
21. Hermans, I. F., Silk, J. D., Gileadi, U., Masri, S. H., Shepherd, D., Farrand, K. J., Salio, M. & Cerundolo, V. Dendritic Cell Function Can Be Modulated through Cooperative Actions of TLR Ligands and Invariant NKT Cells. *J. Immunol.* **178**, 2721–2729 (2007).
22. Kenna, T., Mason, L. G., Porcelli, S. A., Koezuka, Y., Hegarty, J. E., O'Farrelly, C. & Doherty, D. G. NKT Cells from Normal and Tumor-Bearing Human Livers

Are Phenotypically and Functionally Distinct from Murine NKT Cells. *J. Immunol.* **171**, 1775–1779 (2003).

23. Rahimpour, A., Koay, H. F., Enders, A., Clanchy, R., Eckle, S. B. G., Meehan, B., Chen, Z., Whittle, B., Liu, L., Fairlie, D. P., Goodnow, C. C., McCluskey, J., Rossjohn, J., Uldrich, A. P., Pellicci, D. G. & Godfrey, D. I. Identification of phenotypically and functionally heterogeneous mouse mucosal-associated invariant T cells using MR1 tetramers. *J. Exp. Med.* **212**, 1095–1108 (2015).
24. Salou, M., Legoux, F., Gilet, J., Darbois, A., Du Halgouet, A., Alonso, R., Richer, W., Goubet, A. G., Daviaud, C., Menger, L., Procopio, E., Premel, V. & Lantz, O. A common transcriptomic program acquired in the thymus defines tissue residency of MAIT and NKT subsets. *J. Exp. Med.* **216**, 133–151 (2019).
25. Dusseaux, M., Martin, E., Serriari, N., Péguillet, I., Premel, V., Louis, D., Milder, M., Le Bourhis, L., Soudais, C., Treiner, E. & Lantz, O. Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161 hi IL-17-secreting T cells. *Blood* **117**, 1250–1259 (2011).
26. Meierovics, A., Yankelevich, W. J. C. & Cowley, S. C. MAIT cells are critical for optimal mucosal immune responses during in vivo pulmonary bacterial infection. *Proc. Natl. Acad. Sci. U. S. A.* **110**, E3119-28 (2013).
27. Meierovics, A. I. & Cowley, S. C. MAIT cells promote inflammatory monocyte differentiation into dendritic cells during pulmonary intracellular infection. *J. Exp. Med.* **213**, 2793–2809 (2016).
28. Murayama, G., Chiba, A., Suzuki, H., Nomura, A., Mizuno, T., Kuga, T., Nakamura, S., Amano, H., Hirose, S., Yamaji, K., Suzuki, Y., Tamura, N. & Miyake, S. A Critical Role for Mucosal-Associated Invariant T Cells as Regulators and Therapeutic Targets in Systemic Lupus Erythematosus. *Front. Immunol.* **10**, 2681 (2019).
29. Salio, M., Gasser, O., Gonzalez-Lopez, C., Martens, A., Veerapen, N., Gileadi, U., Verter, J. G., Napolitani, G., Anderson, R., Painter, G., Besra, G. S., Hermans, I. F. & Cerundolo, V. Activation of Human Mucosal-Associated Invariant T Cells Induces CD40L-Dependent Maturation of Monocyte-Derived and Primary Dendritic Cells. *J. Immunol.* **199**, 2631–2638 (2017).
30. Medzhitov, R. & Janeway, C. A. Decoding the patterns of self and nonself by the innate immune system. *Science* vol. 296 298–300 (2002).
31. Germain, R. N. MHC-dependent antigen processing and peptide presentation:

Providing ligands for T lymphocyte activation. *Cell* **76**, 287–299 (1994).

32. Watts, C. Capture and Processing of Exogenous Antigens for Presentation on Mhc Molecules. *Annu. Rev. Immunol.* **15**, 821–850 (1997).
33. Henry, R. M., Hoppe, A. D., Joshi, N. & Swanson, J. A. The uniformity of phagosome maturation in macrophages. *J. Cell Biol.* **164**, 185–194 (2004).
34. Blander, J. M. & Medzhitov, R. On regulation of phagosome maturation and antigen presentation. *Nat. Immunol.* **7**, 1029–1035 (2006).
35. Shi, G. P., Munger, J. S., Meara, J. P., Rich, D. H. & Chapman, H. A. Molecular cloning and expression of human alveolar macrophage cathepsin S, an elastinolytic cysteine protease. *J. Biol. Chem.* **267**, 7258–7262 (1992).
36. Guo-Ping, S., Villadangos, J. A., Dranoff, G., Small, C., Lijuan, G., Haley, K. J., Riese, R., Ploegh, H. L. & Chapman, H. A. Cathepsin S required for normal MHC class II peptide loading and germinal center development. *Immunity* **10**, 197–206 (1999).
37. Busch, R., Reich, Z., Zaller, D. M., Sloan, V. & Mellins, E. D. Secondary structure composition and pH-dependent conformational changes of soluble recombinant HLA-DM. *J. Biol. Chem.* **273**, 27557–27564 (1998).
38. Ciechanover, A. The ubiquitin-proteasome proteolytic pathway. *Cell* vol. 79 13–21 (1994).
39. Daar, A. S., Fuggle, S. V., Fabre, J. W., Ting, A. & Morris, P. J. The detailed distribution of HLA–A, B, C antigens in normal human organs. *Transplantation* **38**, 287–292 (1984).
40. Lévy, F., Burri, L., Morel, S., Peitrequin, A.-L., Lévy, N., Bachi, A., Hellman, U., Van den Eynde, B. J. & Servis, C. The Final N-Terminal Trimming of a Subaminoterminal Proline-Containing HLA Class I-Restricted Antigenic Peptide in the Cytosol Is Mediated by Two Peptidases. *J. Immunol.* **169**, 4161–4171 (2002).
41. Shen, X. Z., Billet, S., Lin, C., Okwan-Duodu, D., Chen, X., Lukacher, A. E. & Bernstein, K. E. The carboxypeptidase ACE shapes the MHC class i peptide repertoire. *Nat. Immunol.* **12**, 1078–1085 (2011).
42. Trombetta, E. S., Ebersold, M., Garrett, W., Pypaert, M. & Mellman, I. Activation of lysosomal function during dendritic cell maturation. *Science (80-.).* **299**, 1400–1403 (2003).
43. Savina, A., Jancic, C., Hugues, S., Guermonprez, P., Vargas, P., Moura, I. C., Lennon-Duménil, A. M., Seabra, M. C., Raposo, G. & Amigorena, S. NOX2

Controls Phagosomal pH to Regulate Antigen Processing during Crosspresentation by Dendritic Cells. *Cell* **126**, 205–218 (2006).

44. Kovacsovics-Bankowski, M. & Rock, K. L. A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. *Science (80-.).* **267**, 243–246 (1995).
45. Pfeifer, J. D., Wick, M. J., Roberts, R. L., Findlay, K., Normark, S. J. & Harding, C. V. Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. *Nature* **361**, 359–362 (1993).
46. Belizaire, R. & Unanue, E. R. Targeting proteins to distinct subcellular compartments reveals unique requirements for MHC class I and II presentation. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 17463–17468 (2009).
47. Shen, L., Sigal, L. J., Boes, M. & Rock, K. L. Important role of cathepsin S in generating peptides for TAP-independent MHC class I crosspresentation in vivo. *Immunity* **21**, 155–165 (2004).
48. Di Pucchio, T., Chatterjee, B., Smed-Sörensen, A., Clayton, S., Palazzo, A., Montes, M., Xue, Y., Mellman, I., Banchereau, J. & Connolly, J. E. Direct proteasome-independent cross-presentation of viral antigen by plasmacytoid dendritic cells on major histocompatibility complex class I. *Nat. Immunol.* **9**, 551–557 (2008).
49. Mellman, I. & Steinman, R. M. Dendritic cells: Specialized and regulated antigen processing machines. *Cell* vol. 106 255–258 (2001).
50. Rodríguez-Pinto, D. B cells as antigen presenting cells. *Cellular Immunology* vol. 238 67–75 (2005).
51. Cannon, G. J. & Swanson, J. A. The macrophage capacity for phagocytosis. *J. Cell Sci.* **101**, 907–913 (1992).
52. Prickett, T. C., McKenzie, J. L. & Hart, D. N. J. Characterization of interstitial dendritic cells in human liver. *Transplantation* **46**, 754–761 (1988).
53. Hart, D. N. J. & McKenzie, J. L. Isolation and characterization of human tonsil dendritic cells. *J. Exp. Med.* **168**, 157–170 (1988).
54. Lenz, A., Heine, M., Schuler, G. & Romani, N. Human and murine dermis contain dendritic cells: Isolation by means of a novel method and phenotypical and functional characterization. *J. Clin. Invest.* **92**, 2587–2596 (1993).
55. Jaensson, E., Uronen-Hansson, H., Pabst, O., Eksteen, B., Tian, J., Coombes, J. L., Berg, P. L., Davidsson, T., Powrie, F., Johansson-Lindbom, B. & Agace, W. W.

Small intestinal CD103+ dendritic cells display unique functional properties that are conserved between mice and humans. *J. Exp. Med.* **205**, 2139–2149 (2008).

56. Mcllroy, D., Troadec, C., Grassi, F., Samri, A., Barrou, B., Autran, B., Debré, P., Feuillard, J. & Hosmalin, A. Investigation of human spleen dendritic cell phenotype and distribution reveals evidence of in vivo activation in a subset of organ donors. *Blood* **97**, 3470–3477 (2001).
57. León, B., López-Bravo, M. & Ardavín, C. Monocyte-Derived Dendritic Cells Formed at the Infection Site Control the Induction of Protective T Helper 1 Responses against Leishmania. *Immunity* **26**, 519–531 (2007).
58. Wilson, N. S., El-Sukkari, D., Belz, G. T., Smith, C. M., Steptoe, R. J., Heath, W. R., Shortman, K. & Villadangos, J. A. Most lymphoid organ dendritic cell types are phenotypically and functionally immature. *Blood* **102**, 2187–2194 (2003).
59. Ohl, L., Mohaupt, M., Czeloth, N., Hintzen, G., Kiafard, Z., Zwirner, J., Blankenstein, T., Henning, G. & Förster, R. CCR7 governs skin dendritic cell migration under inflammatory and steady-state conditions. *Immunity* **21**, 279–288 (2004).
60. Wilson, N. S., Young, L. J., Kupresanin, F., Naik, S. H., Vremec, D., Heath, W. R., Akira, S., Shortman, K., Boyle, J., Maraskovsky, E., Belz, G. T. & Villadangos, J. A. Normal proportion and expression of maturation markers in migratory dendritic cells in the absence of germs or Toll-like receptor signaling. *Immunol. Cell Biol.* **86**, 200–205 (2008).
61. Ginhoux, F., Liu, K., Helft, J., Bogunovic, M., Greter, M., Hashimoto, D., Price, J., Yin, N., Bromberg, J., Lira, S. A., Stanley, E. R., Nussenzweig, M. & Merad, M. The origin and development of nonlymphoid tissue CD103+ DCs. *J. Exp. Med.* **206**, 3115–3130 (2009).
62. Edelson, B. T., Wumesh, K. C., Juang, R., Kohyama, M., Benoit, L. A., Klekotka, P. A., Moon, C., Albring, J. C., Ise, W., Michael, D. G., Bhattacharya, D., Stappenbeck, T. S., Holtzman, M. J., Sung, S. S. J., Murphy, T. L., Hildner, K. & Murphy, K. M. Peripheral CD103+ dendritic cells form a unified subset developmentally related to CD8α+ conventional dendritic cells. *J. Exp. Med.* **207**, 823–836 (2010).
63. Pooley, J. L., Heath, W. R. & Shortman, K. Cutting Edge: Intravenous Soluble Antigen Is Presented to CD4 T Cells by CD8 − Dendritic Cells, but Cross-Presented to CD8 T Cells by CD8 + Dendritic Cells . *J. Immunol.* **166**, 5327–5330

(2001).

64. Schnorrer, P., Behrens, G. M. N., Wilson, N. S., Pooley, J. L., Smith, C. M., El-Sukkari, D., Davey, G., Kupresanin, F., Li, M., Maraskovsky, E., Belz, G. T., Carbone, F. R., Shortman, K., Heath, W. R. & Villadangos, J. A. The dominant role of CD8+ dendritic cells in cross-presentation is not dictated by antigen capture. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 10729–10734 (2006).

65. Hildner, K., Edelson, B. T., Purtha, W. E., Diamond, M., Matsushita, H., Kohyama, M., Calderon, B., Schraml, B. U., Unanue, E. R., Diamond, M. S., Schreiber, R. D., Murphy, T. L. & Murphy, K. M. Batf3 deficiency reveals a critical role for CD8α+ dendritic cells in cytotoxic T cell immunity. *Science (80-.).* **322**, 1097–1100 (2008).

66. Mahnke, K., Guo, M., Lee, S., Sepulveda, H., Swain, S. L., Nussenzweig, M. & Steinman, R. M. The dendritic cell receptor for endocytosis, DEC-205, can recycle and enhance antigen presentation via major histocompatibility complex class II-positive lysosomal compartments. *J. Cell Biol.* **151**, 673–683 (2000).

67. Schreibelt, G., Klinkenberg, L. J. J., Cruz, L. J., Tacken, P. J., Tel, J., Kreutz, M., Adema, G. J., Brown, G. D., Figdor, C. G. & De Vries, I. J. M. The C-type lectin receptor CLEC9A mediates antigen uptake and (cross-)presentation by human blood BDCA3+ myeloid dendritic cells. *Blood* **119**, 2284–2292 (2012).

68. Idoyaga, J., Cheong, C., Suda, K., Suda, N., Kim, J. Y., Lee, H., Park, C. G. & Steinman, R. M. Cutting Edge: Langerin/CD207 Receptor on Dendritic Cells Mediates Efficient Antigen Presentation on MHC I and II Products In Vivo. *J. Immunol.* **180**, 3647–3650 (2008).

69. Hemmi, H., Zaidi, N., Wang, B., Matos, I., Fiorese, C., Lubkin, A., Zbytnuik, L., Suda, K., Zhang, K., Noda, M., Kaisho, T., Steinman, R. M. & Idoyaga, J. Treml4, an Ig Superfamily Member, Mediates Presentation of Several Antigens to T Cells In Vivo, Including Protective Immunity to HER2 Protein. *J. Immunol.* **188**, 1147–1155 (2012).

70. Elpek, K. G., Bellemare-Pelletier, A., Malhotra, D., Reynoso, E. D., Lukacs-Kornek, V., DeKruyff, R. H. & Turley, S. J. Lymphoid organ-resident dendritic cells exhibit unique transcriptional fingerprints based on subset and site. *PLoS One* **6**, (2011).

71. Dorner, B. G., Dorner, M. B., Zhou, X., Opitz, C., Mora, A., Güttler, S., Hutloff, A., Mages, H. W., Ranke, K., Schaefer, M., Jack, R. S., Henn, V. & Kroczek, R.

A. Selective Expression of the Chemokine Receptor XCR1 on Cross-presenting Dendritic Cells Determines Cooperation with CD8+ T Cells. *Immunity* **31**, 823–833 (2009).

72. Lewis, K. L., Caton, M. L., Bogunovic, M., Greter, M., Grajkowska, L. T., Ng, D., Klinakis, A., Charo, I. F., Jung, S., Gommerman, J. L., Ivanov, I. I., Liu, K., Merad, M. & Reizis, B. Notch2 receptor signaling controls functional differentiation of dendritic cells in the spleen and intestine. *Immunity* **35**, 780–791 (2011).
73. Tussiwand, R., Everts, B., Grajales-Reyes, G. E., Kretzer, N. M., Iwata, A., Bagaitkar, J., Wu, X., Wong, R., Anderson, D. A., Murphy, T. L., Pearce, E. J. & Murphy, K. M. Klf4 Expression in Conventional Dendritic Cells Is Required for T Helper 2 Cell Responses. *Immunity* **42**, 916–928 (2015).
74. Bajaña, S., Roach, K., Turner, S., Paul, J. & Kovats, S. IRF4 Promotes Cutaneous Dendritic Cell Migration to Lymph Nodes during Homeostasis and Inflammation. *J. Immunol.* **189**, 3368–3377 (2012).
75. Vander Lugt, B., Khan, A. A., Hackney, J. A., Agrawal, S., Lesch, J., Zhou, M., Lee, W. P., Park, S., Xu, M., Devoss, J., Spooner, C. J., Chalouni, C., Delamarre, L., Mellman, I. & Singh, H. Transcriptional programming of dendritic cells for enhanced MHC class II antigen presentation. *Nat. Immunol.* **15**, 161–167 (2014).
76. Maldonado-López, R. & Moser, M. Dendritic cell subsets and the regulation of Th1/Th2 responses. in *Seminars in Immunology* vol. 13 275–282 (Elsevier, 2001).
77. Mathers, A. R., Janelsins, B. M., Rubin, J. P., Tkacheva, O. A., Shufesky, W. J., Watkins, S. C., Morelli, A. E. & Larregina, A. T. Differential Capability of Human Cutaneous Dendritic Cell Subsets to Initiate Th17 Responses. *J. Immunol.* **182**, 921–933 (2009).
78. Plantinga, M., Guilliams, M., Vanheerswynghels, M., Deswarte, K., Branco-Madeira, F., Toussaint, W., Vanhoutte, L., Neyt, K., Killeen, N., Malissen, B., Hammad, H. & Lambrecht, B. N. Conventional and Monocyte-Derived CD11b+ Dendritic Cells Initiate and Maintain T Helper 2 Cell-Mediated Immunity to House Dust Mite Allergen. *Immunity* **38**, 322–335 (2013).
79. Tamoutounour, S., Henri, S., Lelouard, H., de Bovis, B., de Haar, C., van der Woude, C. J., Woltman, A. M., Reyal, Y., Bonnet, D., Sichien, D., Bain, C. C., Mowat, A. M., Reis e Sousa, C., Poulin, L. F., Malissen, B. & Guilliams, M. CD64 distinguishes macrophages from dendritic cells in the gut and reveals the Th1-inducing role of mesenteric lymph node macrophages during colitis. *Eur. J.*

Immunol. **42**, 3150–3166 (2012).

80. Schreiber, H. A., Loschko, J., Karssemeijer, R. A., Escolano, A., Meredith, M. M., Mucida, D., Guermonprez, P. & Nussenzweig, M. C. Intestinal monocytes and macrophages are required for T cell polarization in response to Citrobacter rodentium. *J. Exp. Med.* **210**, 2025–2039 (2013).
81. Zhan, Y., Xu, Y., Seah, S., Brady, J. L., Carrington, E. M., Cheers, C., Croker, B. A., Wu, L., Villadangos, J. A. & Lew, A. M. Resident and Monocyte-Derived Dendritic Cells Become Dominant IL-12 Producers under Different Conditions and Signaling Pathways. *J. Immunol.* **185**, 2125–2133 (2010).
82. Kohrgruber, N., Gröger, M., Meraner, P., Kriehuber, E., Petzelbauer, P., Brandt, S., Stingl, G., Rot, A. & Maurer, D. Plasmacytoid Dendritic Cell Recruitment by Immobilized CXCR3 Ligands. *J. Immunol.* **173**, 6592–6602 (2004).
83. Nakano, H., Yanagita, M. & Gunn, M. D. CD11c+B220+Gr-1+ cells in mouse lymph nodes and spleen display characteristics of plasmacytoid dendritic cells. *J. Exp. Med.* **194**, 1171–1178 (2001).
84. Yoneyama, H., Matsuno, K., Zhang, Y., Nishiwaki, T., Kitabatake, M., Ueha, S., Narumi, S., Morikawa, S., Ezaki, T., Lu, B., Gerard, C., Ishikawa, S. & Matsushima, K. Evidence for recruitment of plasmacytoid dendritic cell precursors to inflamed lymph nodes through high endothelial venules. *Int. Immunol.* **16**, 915–928 (2004).
85. Farkas, L., Beiske, K., Lund-Johansen, F., Brandtzaeg, P. & Jahnsen, F. L. Plasmacytoid dendritic cells (natural interferon-α/β-producing cells) accumulate in cutaneous lupus erythematosus lesions. *Am. J. Pathol.* **159**, 237–243 (2001).
86. Lande, R., Giacomini, E., Serafini, B., Rosicarelli, B., Sebastiani, G. D., Minisola, G., Tarantino, U., Riccieri, V., Valesini, G. & Coccia, E. M. Characterization and Recruitment of Plasmacytoid Dendritic Cells in Synovial Fluid and Tissue of Patients with Chronic Inflammatory Arthritis. *J. Immunol.* **173**, 2815–2824 (2004).
87. Kadowaki, N., Antonenko, S. & Liu, Y.-J. Distinct CpG DNA and Polyinosinic-Polycytidylic Acid Double-Stranded RNA, Respectively, Stimulate CD11c − Type 2 Dendritic Cell Precursors and CD11c + Dendritic Cells to Produce Type I IFN . *J. Immunol.* **166**, 2291–2295 (2001).
88. Gilliet, M., Cao, W. & Liu, Y. J. Plasmacytoid dendritic cells: Sensing nucleic acids in viral infection and autoimmune diseases. *Nature Reviews Immunology* vol. 8 594–606 (2008).

89. Dai, J., Megjugorac, N. J., Amrute, S. B. & Fitzgerald-Bocarsly, P. Regulation of IFN Regulatory Factor-7 and IFN-α Production by Enveloped Virus and Lipopolysaccharide in Human Plasmacytoid Dendritic Cells. *J. Immunol.* **173**, 1535–1548 (2004).
90. Cella, M., Facchetti, F., Lanzavecchia, A. & Colonna, M. Plasmacytoid dendritic cells activated by influenza virus and CD40L drive a potent TH1 polarization. *Nat. Immunol.* **1**, 305–310 (2000).
91. Grouard, G., Rissoan, M. C., Filgueira, L., Durand, I., Banchereau, J. & Liu, Y. J. The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. *J. Exp. Med.* **185**, 1101–1111 (1997).
92. Sapoznikov, A., Fischer, J. A. A., Zaft, T., Krauthgamer, R., Dzionek, A. & Jung, S. Organ-dependent in vivo priming of naive CD4+, but not CD8 +, T cells by plasmacytoid dendritic cells. *J. Exp. Med.* **204**, 1923–1933 (2007).
93. Jaehn, P. S., Zaenker, K. S., Schmitz, J. & Dzionek, A. Functional dichotomy of plasmacytoid dendritic cells: Antigen-specific activation of T cells versus production of type I interferon. *Eur. J. Immunol.* **38**, 1822–1832 (2008).
94. Salio, M., Palmowski, M. J., Atzberger, A., Hermans, I. F. & Cerundolo, V. CpG-matured Murine Plasmacytoid Dendritic Cells Are Capable of In Vivo Priming of Functional CD8 T Cell Responses to Endogenous but Not Exogenous Antigens. *J. Exp. Med.* **199**, 567–579 (2004).
95. Inaba, K., Turley, S., Yamaide, F., Iyoda, T., Mahnke, K., Inaba, M., Pack, M., Subklewe, M., Sauter, B., Sheff, D., Albert, M., Bhardwaj, N., Mellman, I. & Steinman, R. M. Efficient presentation of phagocytosed cellular fragments on the major histocompatibility complex class II products of dendritic cells. *J. Exp. Med.* **188**, 2163–2173 (1998).
96. Pribila, J. T., Quale, A. C., Mueller, K. L. & Shimizu, Y. Integrins and T Cell–Mediated Immunity. *Annu. Rev. Immunol.* **22**, 157–180 (2004).
97. Luster, A. D. The role of chemokines in linking innate and adaptive immunity. *Current Opinion in Immunology* vol. 14 129–135 (2002).
98. Sallusto, F. & Lanzavecchia, A. Understanding dendritic cell and T-lymphocyte traffic through the analysis of chemokine receptor expression. *Immunological Reviews* vol. 177 134–140 (2000).
99. Pasare, C. & Medzhitov, R. Toll-like receptors: Linking innate and adaptive immunity. in *Advances in Experimental Medicine and Biology* vol. 560 11–18

(2005).

100. Lin, S. C., Lo, Y. C. & Wu, H. Helical assembly in the MyD88-IRAK4-IRAK2 complex in TLR/IL-1R signalling. *Nature* **465**, 885–890 (2010).

101. Chang, M., Jin, W. & Sun, S. C. Peli1 facilitates TRIF-dependent Toll-like receptor signaling and proinflammatory cytokine production. *Nat. Immunol.* **10**, 1089–1095 (2009).

102. Iwasaki, A. & Medzhitov, R. Toll-like receptor control of the adaptive immune responses. *Nature Immunology* vol. 5 987–995 (2004).

103. Radtke, F., Wilson, A., Stark, G., Bauer, M., Van Meerwijk, J., MacDonald, H. R. & Aguet, M. Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity* **10**, 547–558 (1999).

104. Pui, J. C., Allman, D., Xu, L., DeRocco, S., Karnell, F. G., Bakkour, S., Lee, J. Y., Kadesch, T., Hardy, R. R., Aster, J. C. & Pear, W. S. Notch1 expression in early lymphopoiesis influences B versus T lineage determination. *Immunity* **11**, 299–308 (1999).

105. Krangel, M. S. Mechanics of T cell receptor gene rearrangement. *Current Opinion in Immunology* vol. 21 133–139 (2009).

106. Hernández-Hoyos, G., Sohn, S. J., Rothenberg, E. V. & Alberola-Ila, J. Lck activity controls CD4/CD8 T cell lineage commitment. *Immunity* **12**, 313–322 (2000).

107. Yasutomo, K., Doyle, C., Miele, L. & Germain, R. N. The duration of antigen receptor signalling determines CD4+ versus CD8+ T-cell lineage fate. *Nature* **404**, 506–510 (2000).

108. Jenne, L., Arrighi, J. F., Jonuleit, H., Saurat, J. H. & Hauser, C. Dendritic cells containing apoptotic melanoma cells prime human CD8+ T cells for efficient tumor cell lysis. *Cancer Res.* **60**, 4446–4452 (2000).

109. Ohminami, H., Yasukawa, M. & Fujita, S. HLA class I-restricted lysis of leukemia cells by a CD8+ cytotoxic T- lymphocyte clone specific for WT1 peptide. *Blood* **95**, 286–293 (2000).

110. Kägi, D., Ledermann, B., Bürki, K., Hengartner, H. & Zinkernagel, R. M. CD8+ T cell-mediated protection against an intracellular bacterium by perforin-dependent cytotoxicity. *Eur. J. Immunol.* **24**, 3068–3072 (1994).

111. Zhou, L., Chong, M. M. W. & Littman, D. R. Plasticity of CD4+ T Cell Lineage Differentiation. *Immunity* vol. 30 646–655 (2009).

112. Shedlock, D. J. & Shen, H. Requirement for CD4 T cell help in generating functional CD8 T cell memory. *Science (80-.).* **300**, 337–339 (2003).

113. Lugo-Villarino, G., Maldonado-López, R., Possemato, R., Peñaranda, C. & Glimcher, L. H. T-bet is required for optimal production of IFN-γ and antigen-specific T cell activation by dendritic cells. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 7749–7754 (2003).

114. Ahonen, C. L., Doxsee, C. L., McGurran, S. M., Riter, T. R., Wade, W. F., Barth, R. J., Vasilakos, J. P., Noelle, R. J. & Kedl, R. M. Combined TLR and CD40 Triggering Induces Potent CD8+ T Cell Expansion with Variable Dependence on Type I IFN. *J. Exp. Med.* **199**, 775–784 (2004).

115. Salmond, R. J., Filby, A., Qureshi, I., Caserta, S. & Zamoyska, R. T-cell receptor proximal signaling via the Src-family kinases, Lck and Fyn, influences T-cell activation, differentiation, and tolerance. *Immunol. Rev.* **228**, 9–22 (2009).

116. Sharpe, A. H. & Freeman, G. J. The B7-CD28 superfamily. *Nature Reviews Immunology* vol. 2 116–126 (2002).

117. Curtsinger, J. M., Schmidt, C. S., Mondino, A., Lins, D. C., Kedl, R. M., Jenkins, M. K. & Mescher, M. F. Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells. *J. Immunol.* **162**, 3256–62 (1999).

118. Curtsinger, J. M., Johnson, C. M. & Mescher, M. F. CD8 T Cell Clonal Expansion and Development of Effector Function Require Prolonged Exposure to Antigen, Costimulation, and Signal 3 Cytokine. *J. Immunol.* **171**, 5165–5171 (2003).

119. Bromley, S. K., Thomas, S. Y. & Luster, A. D. Chemokine receptor CCR7 guides T cell exit from peripheral tissues and entry into afferent lymphatics. *Nat. Immunol.* **6**, 895–901 (2005).

120. Haluszczak, C., Akue, A. D., Hamilton, S. E., Johnson, L. D. S., Pujanauski, L., Teodorovic, L., Jameson, S. C. & Kedl, R. M. The antigen-specific CD8 + T cell repertoire in unimmunized mice includes memory phenotype cells bearing markers of homeostatic expansion. *J. Exp. Med.* **206**, 435–448 (2009).

121. Chouaib, S., Chehimi, J., Bani, L., Genetet, N., Tursz, T., Gay, F., Trinchieri, G. & Mami-Chouaib, F. Interleukin 12 induces the differentiation of major histocompatibility complex class I-primed cytotoxic T-lymphocyte precursors into allospecific cytotoxic effectors. *Proc. Natl. Acad. Sci. U. S. A.* **91**, 12659–12663 (1994).

122. Trapani, J. A. & Smyth, M. J. Functional significance of the perforin/granzyme

cell death pathway. *Nat. Rev. Immunol.* **2**, 735–747 (2002).

123. Slifka, M. K. & Whitton, J. L. Antigen-specific regulation of T cell-mediated cytokine production. *Immunity* vol. 12 451–457 (2000).

124. Badovinac, V. P., Porter, B. B. & Harty, J. T. Programmed contraction of cd8+ t cells after infection. *Nat. Immunol.* **3**, 619–626 (2002).

125. Razvi, E. S. & Welsh, R. M. Programmed cell death of T lymphocytes during acute viral infection: a mechanism for virus-induced immune deficiency. *J. Virol.* **67**, 5754–5765 (1993).

126. Kaech, S. M., Tan, J. T., Wherry, E. J., Konieczny, B. T., Surh, C. D. & Ahmed, R. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat. Immunol.* **4**, 1191–1198 (2003).

127. Huster, K. M., Busch, V., Schiemann, M., Linkemann, K., Kerksiek, K. M., Wagner, H. & Busch, D. H. Selective expression of IL-7 receptor on memory T cells identifies early CD40L-dependent generation of distinct CD8+ memory T cell subsets. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 5610–5615 (2004).

128. Butz, E. A. & Bevan, M. J. Massive expansion of antigen-specific CD8+ T cells during an acute virus infection. *Immunity* **8**, 167–175 (1998).

129. Johnson, E. D. & Cole, G. A. Functional heterogeneity of lymphocytic choriomeningitis virus specific T lymphocytes. I. Identification of effector and memory subsets. *J. Exp. Med.* **141**, 866–881 (1975).

130. Thimme, R., Wieland, S., Steiger, C., Ghrayeb, J., Reimann, K. A., Purcell, R. H. & Chisari, F. V. CD8+ T Cells Mediate Viral Clearance and Disease Pathogenesis during Acute Hepatitis B Virus Infection. *J. Virol.* **77**, 68–76 (2003).

131. Callan, M. F. C., Tan, L., Annels, N., Ogg, G. S., Wilson, J. D. K., O'Callaghan, C. A., Steven, N., McMichael, A. J. & Rickinson, A. B. Direct visualization of antigen-specific CD8+ T cells during the primary immune response to Epstein-Barr virus in vivo. *J. Exp. Med.* **187**, 1395–1402 (1998).

132. Gamadia, L. E., Rentenaar, R. J., Baars, P. A., Remmerswaal, E. B. M., Surachno, S., Weel, J. F. L., Toebes, M., Schumacher, T. N. M., Ten Berge, I. J. M. & Van Lier, R. A. W. Differentiation of cytomegalovirus-specific CD8 + T cells in healthy and immunosuppressed virus carriers. *Blood* **98**, 754–761 (2001).

133. Migueles, S. A., Laborico, A. C., Shupert, W. L., Sabbaghian, M. S., Rabin, R., Hallahan, C. W., Van Baarle, D., Kostense, S., Miedema, F., McLaughlin, M., Ehler, L., Metcalf, J., Liu, S. & Connors, M. HIV-specific CD8+ T cell

proliferation is coupled to perforin expression and is maintained in nonprogressors. *Nat. Immunol.* **3**, 1061–1068 (2002).

134. Naito, Y., Saito, K., Shiiba, K., Ohuchi, A., Saigenji, K., Nagura, H. & Ohtani, H. CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. *Cancer Res.* **58**, 3491–3494 (1998).
135. Sato, E., Olson, S. H., Ahn, J., Bundy, B., Nishikawa, H., Qian, F., Jungbluth, A. A., Frosina, D., Gnjatic, S., Ambrosone, C., Kepner, J., Odunsi, T., Ritter, G., Lele, S., Chen, Y. T., Ohtani, H., Old, L. J. & Odunsi, K. Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 18538–18543 (2005).
136. Mahmoud, S. M. A., Paish, E. C., Powe, D. G., Macmillan, R. D., Grainge, M. J., Lee, A. H. S., Ellis, I. O. & Green, A. R. Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer. *J. Clin. Oncol.* **29**, 1949–1955 (2011).
137. Gooden, M. J. M., De Bock, G. H., Leffers, N., Daemen, T. & Nijman, H. W. The prognostic influence of tumour-infiltrating lymphocytes in cancer: A systematic review with meta-analysis. *British Journal of Cancer* vol. 105 93–103 (2011).
138. Afkarian, M., Sedy, J. R., Yang, J., Jacobson, N. G., Cereb, N., Yang, S. Y., Murphy, T. L. & Murphy, K. M. T-bet is a STATI-induced regulator for IL-12R expression in naïve CD4+ T cells. *Nat. Immunol.* **3**, 549–557 (2002).
139. Murray, H. W., Rubin, B. Y., Carriero, S. M., Harris, A. M. & Jaffee, E. A. Human mononuclear phagocyte antiprotozoal mechanisms: Oxygen-dependent vs oxygen-independent activity against intracellular Toxoplasma gondii. *J. Immunol.* **134**, 1982–1988 (1985).
140. Glimcher, L. H. & Murphy, K. M. Lineage commitment in the immune system: The T helper lymphocyte grows up. *Genes and Development* vol. 14 1693–1711 (2000).
141. Annunziato, F., Cosmi, L., Santarlasci, V., Maggi, L., Liotta, F., Mazzinghi, B., Parente, E., Filì, L., Ferri, S., Frosali, F., Giudici, F., Romagnani, P., Parronchi, P., Tonelli, F., Maggi, E. & Romagnani, S. Phenotypic and functional features of human Th17 cells. *J. Exp. Med.* **204**, 1849–1861 (2007).
142. Weaver, C. T., Harrington, L. E., Mangan, P. R., Gavrieli, M. & Murphy, K. M. Th17: An Effector CD4 T Cell Lineage with Regulatory T Cell Ties. *Immunity* vol. 24 677–688 (2006).

143. Miller, J. F. & Mitchell, G. F. Cell to cell interaction in the immune response. I. Hemolysin-forming cells in neonatally thymectomized mice reconstituted with thymus or thoracic duct lymphocytes. *J. Exp. Med.* **128**, 801–820 (1968).

144. Mitchell, G. F. & Miller, J. F. Cell to cell interaction in the immune response. II. The source of hemolysin-forming cells in irradiated mice given bone marrow and thymus or thoracic duct lymphocytes. *J. Exp. Med.* **128**, 821–837 (1968).

145. Clark, E. A. & Ledbetter, J. A. Activation of human B cells mediated through two distinct cell surface differentiation antigens, Bp35 and Bp50. *Proc. Natl. Acad. Sci. U. S. A.* **83**, 4494–4498 (1986).

146. Armitage, R. J., Fanslow, W. C., Strockbine, L., Sato, T. A., Clifford, K. N., Macduff, B. M., Anderson, D. M., Gimpel, S. D., Davis-Smith, T., Maliszewski, C. R., Clark, E. A., Smith, C. A., Grabstein, K. H., Cosman, D. & Spriggs, M. K. Molecular and biological characterization of a murine ligand for CD40. *Nature* **357**, 80–82 (1992).

147. Tafuri, A., Shahinian, A., Bladt, F., Yoshinaga, S. K., Jordana, M., Wakeham, A., Boucher, L. M., Bouchard, D., Chan, V. S. F., Duncan, G., Odermatt, B., Ho, A., Itie, A., Horan, T., Whoriskey, J. S., Pawson, T., Penninger, J. M., Ohashi, P. S. & Mak, T. W. ICOS is essential for effective T-helper-cell responses. *Nature* **409**, 105–109 (2001).

148. Crotty, S., Kersh, E. N., Cannons, J., Schwartzberg, P. L. & Ahmed, R. SAP is required for generating long term humoral immunity. *Nature* **421**, 282–287 (2003).

149. Aruffo, A., Farrington, M., Hollenbaugh, D., Li, X., Milatovich, A., Nonoyama, S., Bajorath, J., Grosmaire, L. S., Stenkamp, R., Neubauer, M., Roberts, R. L., Noelle, R. J., Ledbetter, J. A., Francke, U. & Ochs, H. D. The CD40 ligand, gp39, is defective in activated T cells from patients with X-linked hyper-IgM syndrome. *Cell* **72**, 291–300 (1993).

150. Pullen, S. S., Dang, T. T. A., Crute, J. J. & Kehry, M. R. CD40 signaling through tumor necrosis factor receptor-associated factors (TRAFs): Binding site specificity and activation of downstream pathways by distinct TRAFs. *J. Biol. Chem.* **274**, 14246–14254 (1999).

151. Hostager, B. S., Haxhinasto, S. A., Rowland, S. L. & Bishop, G. A. Tumor Necrosis Factor Receptor-associated Factor 2 (TRAF2)-deficient B Lymphocytes Reveal Novel Roles for TRAF2 in CD40 Signaling. *J. Biol. Chem.* **278**, 45382–45390 (2003).

152. Jalukar, S. V., Hostager, B. S. & Bishop, G. A. Characterization of the Roles of TNF Receptor-Associated Factor 6 in CD40-Mediated B Lymphocyte Effector Functions. *J. Immunol.* **164**, 623–630 (2000).

153. Jabara, H. H., Laouini, D., Tsitsikov, E., Mizoguchi, E., Bhan, A. K., Castigli, E., Dedeoglu, F., Pivniouk, V., Brodeur, S. R. & Geha, R. S. The Binding Site for TRAF2 and TRAF3 but Not for TRAF6 Is Essential for CD40-Mediated Immunoglobulin Class Switching. *Immunity* **17**, 265–276 (2002).

154. Ahonen, C. L., Manning, E. M., Erickson, L. D., O'Connor, B. P., Lind, E. F., Pullen, S. S., Kehry, M. R. & Noelle, R. J. The CD40-TRAF6 axis controls affinity maturation and the generation of long-lived plasma cells. *Nat. Immunol.* **3**, 451–456 (2002).

155. Ansel, K. M., McHeyzer-Williams, L. J., Ngo, V. N., McHeyzer-Williams, M. G. & Cyster, J. G. In vivo-activated CD4 T cells upregulate CXC chemokine receptor 5 and reprogram their response to lymphoid chemokines. *J. Exp. Med.* **190**, 1123–1134 (1999).

156. King, C., Tangye, S. G. & Mackay, C. R. T Follicular Helper (T FH) Cells in Normal and Dysregulated Immune Responses . *Annu. Rev. Immunol.* **26**, 741–766 (2008).

157. Bennett, S. R. M., Carbone, F. R., Karamalis, F., Miller, J. F. A. P. & Heath, W. R. Induction of a CD8+ cytotoxic T lymphocyte response by cross-priming requires cognate CD4+ T cell help. *J. Exp. Med.* **186**, 65–70 (1997).

158. Keene, J. A. & Forman, J. Helper activity is required for the in vivo generation of cytotoxic T lymphocytes. *J. Exp. Med.* **155**, 768–782 (1982).

159. CASSELL, D. & FORMAN, J. Linked Recognition of Helper and Cytotoxic Antigenic Determinants for the Generation of Cytotoxic T Lymphocytes. *Ann. N. Y. Acad. Sci.* **532**, 51–60 (1988).

160. HUSMANN, L. A. & BEVAN, M. J. Cooperation between Helper T Cells and Cytotoxic T Lymphocyte Precursors. *Ann. N. Y. Acad. Sci.* **532**, 158–169 (1988).

161. Janssen, E. M., Lemmens, E. E., Wolfe, T., Christen, U., Von Herrath, M. G. & Schoenberger, S. P. CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. *Nature* **421**, 852–856 (2003).

162. Sun, J. C. & Bevan, M. J. Defective CD8 T cell memory following acute infection without CD4 T cell help. *Science (80-.).* **300**, 339–342 (2003).

163. Ahrends, T., Babała, N., Xiao, Y., Yagita, H., Van Eenennaam, H. & Borst, J.

CD27 Agonism Plus PD-1 Blockade Recapitulates CD4+ T-cell Help in Therapeutic Anticancer Vaccination. *Cancer Res.* **76**, 2921–2931 (2016).

164. Wherry, E. J. & Kurachi, M. Molecular and cellular insights into T cell exhaustion. *Nature Reviews Immunology* vol. 15 486–499 (2015).
165. Ye, Z., Li, Z., Jin, H. & Qian, Q. Therapeutic Cancer Vaccines. *Advances in experimental medicine and biology* vol. 909 139–167 (2016).
166. Diehl, L., Den Boer, A. T., Schoenberger, S. P., Van Der Voort, E. I. H., Schumacher, T. N. M., Melief, C. J. M., Offringa, R. & Toes, R. E. M. CD40 activation in vivo overcomes peptide-induced peripheral cytotoxic T-lymphocyte tolerance and augments anti-tumor vaccine efficacy. *Nat. Med.* **5**, 774–779 (1999).
167. Beatty, G. L., Li, Y. & Long, K. B. Cancer immunotherapy: activating innate and adaptive immunity through CD40 agonists. *Expert Review of Anticancer Therapy* vol. 17 175–186 (2017).
168. Lantz, O. & Bendelac, A. An invariant T cell receptor α Chain is used by a unique subset of major histocompatibility complex class I-specific CD4+ and CD4-8- T cells in mice and humans. *J. Exp. Med.* **180**, 1077–1106 (1994).
169. Kawano, T., Cui, J., Koezuka, Y., Toura, I., Kaneko, Y., Motoki, K., Ueno, H., Nakagawa, R., Sato, H., Kondo, E., Koseki, H. & Taniguchi, M. CD1d-restricted and TCR-mediated activation of V(α)14 NKT cells by glycosylceramides. *Science (80-.).* **278**, 1626–1629 (1997).
170. Fais, F., Morabito, F., Stelitano, C., Callea, V., Zanardi, S., Scudeletti, M., Varese, P., Ciccone, E. & Grossi, C. E. CD1d is expressed on B-chronic lymphocytic leukemia cells and mediates α-galactosylceramide presentation to natural killer T lymphocytes. *Int. J. Cancer* **109**, 402–411 (2004).
171. Metelitsa, L. S., Weinberg, K. I., Emanuel, P. D. & Seeger, R. C. Expression of CD1d by myelomonocytic leukemias provides a target for cytotoxic NKT cells. *Leukemia* **17**, 1068–1077 (2003).
172. Smyth, M. J., Crowe, N. Y., Pellicci, D. G., Kyparissoudis, K., Kelly, J. M., Takeda, K., Yagita, H. & Godfrey, D. I. Sequential production of interferon-γ by NK1.1+ T cells and natural killer cells is essential for the antimetastatic effect of α-galactosylceramide. *Blood* **99**, 1259–1266 (2002).
173. Crowe, N. Y., Smyth, M. J. & Godfrey, D. I. A critical role for natural killer T cells in immunosurveillance of methylcholanthrene-induced sarcomas. *J. Exp. Med.* **196**, 119–127 (2002).

174. Kitamura, H., Iwakabe, K., Yahata, T., Nishimura, S. I., Ohta, A., Ohmi, Y., Sato, M., Takeda, K., Okumura, K., Van Kaer, L., Kawano, T., Taniguchi, M. & Nishimura, T. The natural killer T (NKT) cell ligand α-galactosylceramide demonstrates its immunopotentiating effect by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells. *J. Exp. Med.* **189**, 1121–1127 (1999).

175. Yang, Y. F., Tomura, M., Ono, S., Hamaoka, T. & Fujiwara, H. Requirement for IFN-γ in IL-12 production induced by collaboration between Vα14+ NKT cells and antigen-presenting cells. *Int. Immunol.* **12**, 1669–1675 (2000).

176. Tomura, M., Yu, W. G., Ahn, H. J., Yamashita, M., Yang, Y. F., Ono, S., Hamaoka, T., Kawano, T., Taniguchi, M., Koezuka, Y. & Fujiwara, H. A novel function of Vα14+CD4+NKT cells: Stimulation of IL-12 production by antigen-presenting cells in the innate immune system. *J. Immunol.* **163**, 93–101 (1999).

177. Bendelac, A., Savage, P. B. & Teyton, L. The Biology of NKT Cells. *Annu. Rev. Immunol.* **25**, 297–336 (2007).

178. Gonzalez-Aseguinolaza, G., De Oliveira, C., Tomaska, M., Hong, S., Bruna-Romero, O., Nakayama, T., Taniguchi, M., Bendelac, A., Van Kaer, L., Koezuka, Y. & Tsuji, M. α-Galactosylceramide-activated Vα14 natural killer T cells mediate protection against murine malaria. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 8461–8466 (2000).

179. Kamijuku, H., Nagata, Y., Jiang, X., Ichinohe, T., Tashiro, T., Mori, K., Taniguchi, M., Hase, K., Ohno, H., Shimaoka, T., Yonehara, S., Odagiri, T., Tashiro, M., Sata, T., Hasegawa, H. & Seino, K. I. Mechanism of NKT cell activation by intranasal coadministration of α-galactosylceramide, which can induce cross-protection against influenza viruses. *Mucosal Immunol.* **1**, 208–218 (2008).

180. Artiaga, B. L., Yang, G., Hackmann, T. J., Liu, Q., Richt, J. A., Salek-Ardakani, S., Castleman, W. L., Lednicky, J. A. & Driver, J. P. α-Galactosylceramide protects swine against influenza infection when administered as a vaccine adjuvant. *Sci. Rep.* **6**, 23593 (2016).

181. Silk, J. D., Hermans, I. F., Gileadi, U., Chong, T. W., Shepherd, D., Salio, M., Mathew, B., Schmidt, R. R., Lunt, S. J., Williams, K. J., Stratford, I. J., Harris, A. L. & Cerundolo, V. Utilizing the adjuvant properties of CD1d-depenclent NKT cells in T cell-mediated immunotherapy. *J. Clin. Invest.* **114**, 1800–1811 (2004).

182. Waldowska, M., Bojarska-Junak, A. & Roliński, J. A brief review of clinical trials

involving manipulation of invariant NKT cells as a promising approach in future cancer therapies. *Cent. Eur. J. Immunol.* **42**, 181–195 (2017).

183. Chang, D. H., Osman, K., Connolly, J., Kukreja, A., Krasovsky, J., Pack, M., Hutchinson, A., Geller, M., Liu, N., Annable, R., Shay, J., Kirchhoff, K., Nishi, N., Ando, Y., Hayashi, K., Hassoun, H., Steinman, R. M. & Dhodapkar, M. V. Sustained expansion of NKT cells and antigen-specific T cells after injection of α-galactosyl-ceramide loaded mature dendritic cells in cancer patients. *J. Exp. Med.* **201**, 1503–1517 (2005).

184. Gasser, O., Sharples, K. J., Barrow, C., Williams, G. M., Bauer, E., Wood, C. E., Mester, B., Dzhelali, M., Caygill, G., Jones, J., Hayman, C. M., Hinder, V. A., Macapagal, J., McCusker, M., Weinkove, R., Painter, G. F., Brimble, M. A., Findlay, M. P., Dunbar, P. R. *et al.* A phase I vaccination study with dendritic cells loaded with NY-ESO-1 and α-galactosylceramide: induction of polyfunctional T cells in high-risk melanoma patients. *Cancer Immunol. Immunother.* **67**, 285–298 (2018).

185. Kjer-Nielsen, L., Patel, O., Corbett, A. J., Le Nours, J., Meehan, B., Liu, L., Bhati, M., Chen, Z., Kostenko, L., Reantragoon, R., Williamson, N. A., Purcell, A. W., Dudek, N. L., McConville, M. J., O'Hair, R. A. J., Khairallah, G. N., Godfrey, D. I., Fairlie, D. P., Rossjohn, J. *et al.* MR1 presents microbial vitamin B metabolites to MAIT cells. *Nature* vol. 491 717–723 (2012).

186. Van Wilgenburg, B., Scherwitzl, I., Hutchinson, E. C., Leng, T., Kurioka, A., Kulicke, C., De Lara, C., Cole, S., Vasanawathana, S., Limpitikul, W., Malasit, P., Young, D., Denney, L., Moore, M. D., Fabris, P., Giordani, M. T., Oo, Y. H., Laidlaw, S. M., Dustin, L. B. *et al.* MAIT cells are activated during human viral infections. *Nat. Commun.* **7**, (2016).

187. Georgel, P., Radosavljevic, M., Macquin, C. & Bahram, S. The non-conventional MHC class I MR1 molecule controls infection by Klebsiella pneumoniae in mice. *Mol. Immunol.* **48**, 769–775 (2011).

188. Bennett, M. S., Trivedi, S., Iyer, A. S., Hale, J. S. & Leung, D. T. Human mucosal-associated invariant T (MAIT) cells possess capacity for B cell help. *J. Leukoc. Biol.* **102**, 1261–1269 (2017).

189. Murayama, G., Chiba, A., Suzuki, H., Nomura, A., Mizuno, T., Kuga, T., Nakamura, S., Amano, H., Hirose, S., Yamaji, K., Suzuki, Y., Tamura, N. & Miyake, S. A Critical Role for Mucosal-Associated Invariant T Cells as Regulators

and Therapeutic Targets in Systemic Lupus Erythematosus. *Frontiers in Immunology* vol. 10 2681 (2019).

190. Koay, H. F., Gherardin, N. A., Enders, A., Loh, L., Mackay, L. K., Almeida, C. F., Russ, B. E., Nold-Petry, C. A., Nold, M. F., Bedoui, S., Chen, Z., Corbett, A. J., Eckle, S. B. G., Meehan, B., D'Udekem, Y., Konstantinov, I. E., Lappas, M., Liu, L., Goodnow, C. C. *et al.* A three-stage intrathymic development pathway for the mucosal-associated invariant T cell lineage. *Nat. Immunol.* **17**, 1300–1311 (2016).
191. Martin, E., Treiner, E., Duban, L., Guerri, L., Laude, H., Toly, C., Premel, V., Devys, A., Moura, I. C., Tilloy, F., Cherif, S., Vera, G., Latour, S., Soudais, C. & Lantz, O. Stepwise development of mait cells in mouse and human. *PLoS Biol.* **7**, 0525–0536 (2009).
192. Tilloy, F., Treiner, E., Park, S. H., Garcia, C., Lemonnier, F., De La Salle, H., Bendelac, A., Bonneville, M. & Lantz, O. An invariant T cell receptor α chain defines a novel TAP-independent major histocompatibility complex class Ib-restricted α/β T cell subpopulation in mammals. *J. Exp. Med.* **189**, 1907–1921 (1999).
193. Treiner, E., Duban, L., Moura, I. C., Hansen, T., Gilfillan, S. & Lantz, O. Mucosal-associated invariant T (MAIT) cells: An evolutionarily conserved T cell subset. *Microbes Infect.* **7**, 552–559 (2005).
194. Savage, A. K., Constantinides, M. G., Han, J., Picard, D., Martin, E., Li, B., Lantz, O. & Bendelac, A. The Transcription Factor PLZF Directs the Effector Program of the NKT Cell Lineage. *Immunity* **29**, 391–403 (2008).
195. Kovalovsky, D., Uche, O. U., Eladad, S., Hobbs, R. M., Yi, W., Alonzo, E., Chua, K., Eidson, M., Kim, H. J., Im, J. S., Pandolfi, P. P. & Sant'Angelo, D. B. The BTB-zinc finger transcriptional regulator PLZF controls the development of invariant natural killer T cell effector functions. *Nat. Immunol.* **9**, 1055–1064 (2008).
196. Constantinides, M. G., McDonald, B. D., Verhoef, P. A. & Bendelac, A. A committed precursor to innate lymphoid cells. *Nature* **508**, 397–401 (2014).
197. Alonzo, E. S., Gottschalk, R. A., Das, J., Egawa, T., Hobbs, R. M., Pandolfi, P. P., Pereira, P., Nichols, K. E., Koretzky, G. A., Jordan, M. S. & Sant'Angelo, D. B. Development of Promyelocytic Zinc Finger and ThPOK-Expressing Innate γδ T Cells Is Controlled by Strength of TCR Signaling and Id3. *J. Immunol.* **184**, 1268–1279 (2010).

198. Salou, M., Legoux, F., Gilet, J., Darbois, A., Du Halgouet, A., Alonso, R., Richer, W., Goubet, A. G., Daviaud, C., Menger, L., Procopio, E., Premel, V. & Lantz, O. A common transcriptomic program acquired in the thymus defines tissue residency of MAIT and NKT subsets. *J. Exp. Med.* **216**, 133–151 (2019).

199. Le Bourhis, L., Dusseaux, M., Bohineust, A., Bessoles, S., Martin, E., Premel, V., Coré, M., Sleurs, D., Serriari, N. E., Treiner, E., Hivroz, C., Sansonetti, P., Gougeon, M. L., Soudais, C. & Lantz, O. MAIT Cells Detect and Efficiently Lyse Bacterially-Infected Epithelial Cells. *PLoS Pathog.* **9**, (2013).

200. Tang, X.-Z., Jo, J., Tan, A. T., Sandalova, E., Chia, A., Tan, K. C., Lee, K. H., Gehring, A. J., De Libero, G. & Bertoletti, A. IL-7 Licenses Activation of Human Liver Intrasinusoidal Mucosal-Associated Invariant T Cells. *J. Immunol.* **190**, 3142–3152 (2013).

201. Hashimoto, K., Okamura, K., Yamaguchi, H., Ototake, M., Nakanishi, T. & Kurosawa, Y. Conservation and diversification of MHC class I and its related molecules in vertebrates. *Immunological Reviews* vol. 167 81–100 (1999).

202. Yamaguchi, H., Hirai, M., Kurosawa, Y. & Hashimoto, K. A highly conserved major histocompatibility complex class I-related gene in mammals. *Biochem. Biophys. Res. Commun.* **238**, 697–702 (1997).

203. Miley, M. J., Truscott, S. M., Yu, Y. Y. L., Gilfillan, S., Fremont, D. H., Hansen, T. H. & Lybarger, L. Biochemical Features of the MHC-Related Protein 1 Consistent with an Immunological Function. *J. Immunol.* **170**, 6090–6098 (2003).

204. Corbett, A. J., Eckle, S. B. G., Birkinshaw, R. W., Liu, L., Patel, O., Mahony, J., Chen, Z., Reantragoon, R., Meehan, B., Cao, H., Williamson, N. A., Strugnell, R. A., Van Sinderen, D., Mak, J. Y. W., Fairlie, D. P., Kjer-Nielsen, L., Rossjohn, J. & McCluskey, J. T-cell activation by transitory neo-antigens derived from distinct microbial pathways. *Nature* **509**, 361–365 (2014).

205. McWilliam, H. E. G., Eckle, S. B. G., Theodossis, A., Liu, L., Chen, Z., Wubben, J. M., Fairlie, D. P., Strugnell, R. A., Mintern, J. D., McCluskey, J., Rossjohn, J. & Villadangos, J. A. The intracellular pathway for the presentation of Vitamin B-related antigens by the antigen-presenting molecule MR1. *Nat. Immunol.* **17**, 531–537 (2016).

206. Patel, O., Kjer-Nielsen, L., Le Nours, J., Eckle, S. B. G., Birkinshaw, R., Beddoe, T., Corbett, A. J., Liu, L., Miles, J. J., Meehan, B., Reantragoon, R., Sandoval-Romero, M. L., Sullivan, L. C., Brooks, A. G., Chen, Z., Fairlie, D. P., McCluskey,

J. & Rossjohn, J. Recognition of vitamin B metabolites by mucosal-associated invariant T cells. *Nat. Commun.* **4**, (2013).

207. Keller, A. N., Eckle, S. B. G., Xu, W., Liu, L., Hughes, V. A., Mak, J. Y. W., Meehan, B. S., Pediongco, T., Birkinshaw, R. W., Chen, Z., Wang, H., D'Souza, C., Kjer-Nielsen, L., Gherardin, N. A., Godfrey, D. I., Kostenko, L., Corbett, A. J., Purcell, A. W., Fairlie, D. P. *et al.* Drugs and drug-like molecules can modulate the function of mucosal-associated invariant T cells. *Nat. Immunol.* **18**, 402–411 (2017).

208. Lepore, M., Kalinichenko, A., Calogero, S., Kumar, P., Paleja, B., Schmaler, M., Narang, V., Zolezzi, F., Poidinger, M., Mori, L. & de Libero, G. Functionally diverse human T cells recognize non-microbial antigens presented by MR1. *Elife* **6**, (2017).

209. Kjer-Nielsen, L., Patel, O., Corbett, A. J., Le Nours, J., Meehan, B., Liu, L., Bhati, M., Chen, Z., Kostenko, L., Reantragoon, R., Williamson, N. A., Purcell, A. W., Dudek, N. L., McConville, M. J., O'Hair, R. A. J., Khairallah, G. N., Godfrey, D. I., Fairlie, D. P., Rossjohn, J. *et al.* MR1 presents microbial vitamin B metabolites to MAIT cells. *Nature* **491**, 717–723 (2012).

210. Mak, J. Y. W., Xu, W., Reid, R. C., Corbett, A. J., Meehan, B. S., Wang, H., Chen, Z., Rossjohn, J., McCluskey, J., Liu, L. & Fairlie, D. P. Stabilizing short-lived Schiff base derivatives of 5-aminouracils that activate mucosal-associated invariant T cells. *Nat. Commun.* **8**, (2017).

211. Li, K., Vorkas, C. K., Chaudhry, A., Bell, D. L., Willis, R. A., Rudensky, A., Altman, J. D., Glickman, M. S. & Aubé, J. Synthesis, stabilization, and characterization of the MR1 ligand precursor 5-amino-6-D-ribitylaminouracil (5-A-RU). *PLoS One* **13**, 20–22 (2018).

212. Yamaguchi, H. & Hashimoto, K. Association of MR1 protein, an MHC class I-related molecule, with β2-microglobulin. *Biochem. Biophys. Res. Commun.* **290**, 722–729 (2002).

213. McWilliam, H. E. G. & Villadangos, J. A. How MR1 Presents a Pathogen Metabolic Signature to Mucosal-Associated Invariant T (MAIT) Cells. *Trends Immunol.* **38**, 679–689 (2017).

214. Harriff, M. J., Karamooz, E., Burr, A., Grant, W. F., Canfield, E. T., Sorensen, M. L., Moita, L. F. & Lewinsohn, D. M. Endosomal MR1 Trafficking Plays a Key Role in Presentation of Mycobacterium tuberculosis Ligands to MAIT Cells. *PLoS*

Pathog. **12**, e1005524 (2016).

215. Karamooz, E., Harriff, M. J., Narayanan, G. A., Worley, A. & Lewinsohn, D. M. MR1 recycling and blockade of endosomal trafficking reveal distinguishable antigen presentation pathways between Mycobacterium tuberculosis infection and exogenously delivered antigens. *Sci. Rep.* **9**, 4797 (2019).
216. Le Bourhis, L., Martin, E., Péguillet, I., Guihot, A., Froux, N., Coré, M., Lévy, E., Dusseaux, M., Meyssonnier, V., Premel, V., Ngo, C., Riteau, B., Duban, L., Robert, D., Rottman, M., Soudais, C. & Lantz, O. Antimicrobial activity of mucosal-associated invariant T cells. *Nat. Immunol.* **11**, 701–708 (2010).
217. Gold, M. C., Cerri, S., Smyk-Pearson, S., Cansler, M. E., Vogt, T. M., Delepine, J., Winata, E., Swarbrick, G. M., Chua, W. J., Yu, Y. Y. L., Lantz, O., Cook, M. S., Null, M. D., Jacoby, D. B., Harriff, M. J., Lewinsohn, D. A., Hansen, T. H. & Lewinsohn, D. M. Human mucosal associated invariant T cells detect bacterially infected cells. *PLoS Biol.* **8**, 1–14 (2010).
218. Chua, W. J., Truscott, S. M., Eickhoff, C. S., Blazevic, A., Hoft, D. F. & Hansen, T. H. Polyclonal mucosa-associated invariant T cells have unique innate functions in bacterial infection. *Infect. Immun.* **80**, 3256–3267 (2012).
219. Smith, D. J., Hill, G. R., Bell, S. C. & Reid, D. W. Reduced mucosal associated invariant T-cells are associated with increased disease severity and Pseudomonas aeruginosa infection in cystic fibrosis. *PLoS One* **9**, V (2014).
220. Grimaldi, D., Le Bourhis, L., Sauneuf, B., Dechartres, A., Rousseau, C., Ouaaz, F., Milder, M., Louis, D., Chiche, J. D., Mira, J. P., Lantz, O. & Pène, F. Specific MAIT cell behaviour among innate-like T lymphocytes in critically ill patients with severe infections. *Intensive Care Med.* **40**, 192–201 (2014).
221. Wang, H., Kjer-Nielsen, L., Shi, M., D'Souza, C., Pediongco, T. J., Cao, H., Kostenko, L., Lim, X. Y., Eckle, S. B. G., Meehan, B. S., Zhu, T., Wang, B., Zhao, Z., Mak, J. Y. W., Fairlie, D. P., Teng, M. W. L., Rossjohn, J., Yu, D., de St Groth, B. F. *et al.* IL-23 costimulates antigen-specific MAIT cell activation and enables vaccination against bacterial infection. *Sci. Immunol.* **4**, eaaw0402 (2019).
222. Treiner, E., Duban, L., Bahram, S., Radosavljevic, M., Wanner, V., Tilloy, F., Affaticati, P., Gilfillan, S. & Lantz, O. addendum: Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1. *Nature* **423**, 1018–1018 (2003).
223. Grignon, D. J., Kristiansen, G., Egevad, L., Amin, M. B., Delahunt, B., Srigley, J.

R., Humphrey, P. A., Epstein, J. I., Amin, M. B., Beltran, H., Lotan, T. L., Mosquera, J.-M., Reuter, V. E., Robinson, B. D., Troncoso, P., Rubin, M. A., Bryant, R. J., Hamdy, F. C., Bermejo, C. E. *et al.* A Contemporary Prostate Cancer Grading System: A Validated Alternative to the Gleason Score. *Eur. Urol.* **6**, n/a-n/a (2015).

224. Piva, L., Tetlak, P., Claser, C., Karjalainen, K., Renia, L. & Ruedl, C. Cutting Edge: Clec9A + Dendritic Cells Mediate the Development of Experimental Cerebral Malaria . *J. Immunol.* **189**, 1128–1132 (2012).
225. Hermans, I. F., Silk, J. D., Yang, J., Palmowski, M. J., Gileadi, U., McCarthy, C., Salio, M., Ronchese, F. & Cerundolo, V. The VITAL assay: A versatile fluorometric technique for assessing CTL- and NKT-mediated cytotoxicity against multiple targets in vitro and in vivo. *J. Immunol. Methods* **285**, 25–40 (2004).
226. Laugel, B., Lloyd, A., Meermeier, E. W., Crowther, M. D., Connor, T. R., Dolton, G., Miles, J. J., Burrows, S. R., Gold, M. C., Lewinsohn, D. M. & Sewell, A. K. Engineering of Isogenic Cells Deficient for MR1 with a CRISPR/Cas9 Lentiviral System: Tools To Study Microbial Antigen Processing and Presentation to Human MR1-Restricted T Cells. *J. Immunol.* **197**, 971–982 (2016).
227. Corbett, A. J., Eckle, S. B. G., Birkinshaw, R. W., Liu, L., Patel, O., Mahony, J., Chen, Z., Reantragoon, R., Meehan, B., Cao, H., Williamson, N. A., Strugnell, R. A., Van Sinderen, D., Mak, J. Y. W., Fairlie, D. P., Kjer-Nielsen, L., Rossjohn, J. & McCluskey, J. T-cell activation by transitory neo-antigens derived from distinct microbial pathways. *Nature* **509**, 361–365 (2014).
228. Wild, M. K., Cambiaggi, A., Brown, M. H., Davies, E. A., Ohno, H., Saito, T. & Van Der Merwe, P. A. Dependence of T cell antigen recognition on the dimensions of an accessory receptor-ligand complex. *J. Exp. Med.* **190**, 31–41 (1999).
229. Mak, J. Y. W., Xu, W., Reid, R. C., Corbett, A. J., Meehan, B. S., Wang, H., Chen, Z., Rossjohn, J., McCluskey, J., Liu, L. & Fairlie, D. P. Stabilizing short-lived Schiff base derivatives of 5-aminouracils that activate mucosal-associated invariant T cells. *Nat. Commun.* **8**, 14599 (2017).
230. Brown, G. M. The Biosynthesis of Pteridines. in *Advances in Enzymology and Related Areas of Molecular Biology* vol. 35 35–77 (2006).
231. Li, K., Vorkas, C. K., Chaudhry, A., Bell, D. L., Willis, R. A., Rudensky, A., Altman, J. D., Glickman, M. S. & Aubé, J. Synthesis, stabilization, and characterization of the MR1 ligand precursor 5-amino-6-D-ribitylaminouracil (5-

A-RU). *PLoS One* **13**, (2018).

232. Chari, R. V. J., Miller, M. L. & Widdison, W. C. Antibody-drug conjugates: An emerging concept in cancer therapy. *Angewandte Chemie - International Edition* vol. 53 3796–3827 (2014).
233. Dubowchik, G. M., Firestone, R. A., Padilla, L., Willner, D., Hofstead, S. J., Mosure, K., Knipe, J. O., Lasch, S. J. & Trail, P. A. Cathepsin B-labile dipeptide linkers for lysosomal release of doxorubicin from internalizing immunoconjugates: Model studies of enzymatic drug release and antigen-specific in vitro anticancer activity. *Bioconjug. Chem.* **13**, 855–869 (2002).
234. Conus, S. & Simon, H. U. Cathepsins and their involvement in immune responses. *Swiss Medical Weekly* vol. 140 (2010).
235. Zavašnik-Bergant, T. & Turk, B. Cysteine cathepsins in the immune response. *Tissue Antigens* vol. 67 349–355 (2006).
236. Cushman, M., Yang, D., Kis, K. & Bacher, A. Design, synthesis, and evaluation of 9-D-ribityl-1,3,7-trihydro-2,6,8-purinetrione, a potent inhibitor of riboflavin synthase and lumazine synthase. *J. Org. Chem.* **66**, 8320–8327 (2001).
237. Kwart, H., Spayd, R. W. & Collins, C. J. Evidence for nitrogen migration in the benzilic acid rearrangement of alloxan and derivatives. *Journal of the American Chemical Society* vol. 83 2579–2580 (1961).
238. Ussher, J. E., van Wilgenburg, B., Hannaway, R. F., Ruustal, K., Phalora, P., Kurioka, A., Hansen, T. H., Willberg, C. B., Phillips, R. E. & Klenerman, P. TLR signaling in human antigen-presenting cells regulates MR1-dependent activation of MAIT cells. *Eur. J. Immunol.* **46**, 1600–1614 (2016).
239. Goldman, S. D. B., Funk, R. S., Rajewski, R. A. & Krise, J. P. Mechanisms of amine accumulation in, and egress from, lysosomes. *Bioanalysis* **1**, 1445–1459 (2009).
240. Yan, Y., Jiang, K., Liu, P., Zhang, X., Dong, X., Gao, J., Liu, Q., Barr, M. P., Zhang, Q., Hou, X., Meng, S. & Gong, P. Bafilomycin A1 induces caspase-independent cell death in hepatocellular carcinoma cells via targeting of autophagy and MAPK pathways. *Sci. Rep.* **6**, (2016).
241. Boya, P., González-Polo, R.-A., Casares, N., Perfettini, J.-L., Dessen, P., Larochette, N., Métivier, D., Meley, D., Souquere, S., Yoshimori, T., Pierron, G., Codogno, P. & Kroemer, G. Inhibition of Macroautophagy Triggers Apoptosis. *Mol. Cell. Biol.* **25**, 1025–1040 (2005).

242. Compton, B. J., Tang, C. W., Johnston, K. A., Osmond, T. L., Hayman, C. M., Larsen, D. S., Hermans, I. F. & Painter, G. F. Synthesis and Activity of 6″-Deoxy-6″-thio-α-GalCer and Peptide Conjugates. *Org. Lett.* **17**, 5954–5957 (2015).
243. Anderson, R. J., Compton, B. J., Tang, C. W., Authier-Hall, A., Hayman, C. M., Swinerd, G. W., Kowalczyk, R., Harris, P., Brimble, M. A., Larsen, D. S., Gasser, O., Weinkove, R., Hermans, I. F. & Painter, G. F. NKT cell-dependent glycolipid-peptide vaccines with potent anti-tumour activity. *Chem. Sci.* **6**, 5120–5127 (2015).
244. Ussher, J. E., van Wilgenburg, B., Hannaway, R. F., Ruustal, K., Phalora, P., Kurioka, A., Hansen, T. H., Willberg, C. B., Phillips, R. E. & Klenerman, P. TLR signaling in human antigen-presenting cells regulates MR1-dependent activation of MAIT cells. *Eur. J. Immunol.* **46**, 1600–1614 (2016).
245. Liu, J. & Brutkiewicz, R. R. The Toll-like receptor 9 signalling pathway regulates MR1-mediated bacterial antigen presentation in B cells. *Immunology* **152**, 232–242 (2017).
246. Chen, Z., Wang, H., D'Souza, C., Sun, S., Kostenko, L., Eckle, S. B. G., Meehan, B. S., Jackson, D. C., Strugnell, R. A., Cao, H., Wang, N., Fairlie, D. P., Liu, L., Godfrey, D. I., Rossjohn, J., McCluskey, J. & Corbett, A. J. Mucosal-associated invariant T-cell activation and accumulation after in vivo infection depends on microbial riboflavin synthesis and co-stimulatory signals. *Mucosal Immunol.* **10**, 58–68 (2017).
247. Wang, H., D'Souza, C., Lim, X. Y., Kostenko, L., Pediongco, T. J., Eckle, S. B. G., Meehan, B. S., Shi, M., Wang, N., Li, S., Liu, L., Mak, J. Y. W., Fairlie, D. P., Iwakura, Y., Gunnersen, J. M., Stent, A. W., Godfrey, D. I., Rossjohn, J., Westall, G. P. *et al.* MAIT cells protect against pulmonary Legionella longbeachae infection. *Nat. Commun.* **9**, 3350 (2018).
248. Park, H. Y., Tan, P. S., Kavishna, R., Ker, A., Lu, J., Chan, C. E. Z., Hanson, B. J., MacAry, P. A., Caminschi, I., Shortman, K., Alonso, S. & Lahoud, M. H. Enhancing vaccine antibody responses by targeting Clec9A on dendritic cells. *npj Vaccines* **2**, (2017).
249. Harandi, A. M., Medaglini, D. & Shattock, R. J. Vaccine adjuvants: A priority for vaccine research. *Vaccine* **28**, 2363–2366 (2010).
250. Van Pinxteren, L. A. H., Cassidy, J. P., Smedegaard, B. H. C., Agger, E. M. & Andersen, P. Control of latent Mycobacterium tuberculosis infection is dependent on CD8 T cells. *Eur. J. Immunol.* **30**, 3689–3698 (2000).

251. Temmerman, S. T., Place, S., Debrie, A., Locht, C. & Mascart, F. Effector Functions of Heparin-Binding Hemagglutinin–Specific CD8 + T Lymphocytes in Latent Human Tuberculosis . *J. Infect. Dis.* **192**, 226–232 (2005).

252. Betts, M. R., Nason, M. C., West, S. M., De Rosa, S. C., Migueles, S. A., Abraham, J., Lederman, M. M., Benito, J. M., Goepfert, P. A., Connors, M., Roederer, M. & Koup, R. A. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. *Blood* **107**, 4781–4789 (2006).

253. Hirata, Y., Egea, L., Dann, S., Eckmann, L. & Kagnoff, M. GM-CSF promoted DC recruitment and survival governs the intestinal mucosal response to enteric attaching-and-effacing bacterial pathogens. *Cell Host Microbe* **7**, 151–163 (2010).

254. Van De Laar, L., Coffer, P. J. & Woltman, A. M. Regulation of dendritic cell development by GM-CSF: Molecular control and implications for immune homeostasis and therapy. *Blood* vol. 119 3383–3393 (2012).

255. Münz, C., Steinman, R. M. & Fujii, S. I. Dendritic cell maturation by innate lymphocytes: Coordinated stimulation of innate and adaptive immunity. *Journal of Experimental Medicine* vol. 202 203–207 (2005).

256. Canchis, P. W., Bhan, A. K., Landau, S. B., Yang, L., Balk, S. P. & Blumberg, R. S. Tissue distribution of the non-polymorphic major histocompatibility complex class I-like molecule, CD1d. *Immunology* **80**, 561–5 (1993).

257. Blumberg, R. S., Terhorst, C., Bleicher, P., McDermott, F. V, Allan, C. H., Landau, S. B., Trier, J. S. & Balk, S. P. Expression of a nonpolymorphic MHC class I-like molecule, CD1D, by human intestinal epithelial cells. *J. Immunol.* **147**, 2518–24 (1991).

258. Bendelac, A., Rivera, M. N., Park, S.-H. & Roark, J. H. MOUSE CD1-SPECIFIC NK1 T CELLS: Development, Specificity, and Function. *Annu. Rev. Immunol.* **15**, 535–562 (1997).

259. Anderson, R. J., Tang, C. W., Daniels, N. J., Compton, B. J., Hayman, C. M., Johnston, K. A., Knight, D. A., Gasser, O., Poyntz, H. C., Ferguson, P. M., Larsen, D. S., Ronchese, F., Painter, G. F. & Hermans, I. F. A self-adjuvanting vaccine induces cytotoxic Tlymphocytes that suppress allergy. *Nat. Chem. Biol.* **10**, 943–949 (2014).

260. Lin, K. Y., Guarnieri, F. G., Staveley-O'Carroll, K. F., Levitsky, H. I., August, J. T., Pardoll, D. M. & Wu, T. C. Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor

antigen. *Cancer Res.* **56**, 21–26 (1996).

261. Jung, S., Unutmaz, D., Wong, P., Sano, G. I., De Los Santos, K., Sparwasser, T., Wu, S., Vuthoori, S., Ko, K., Zavala, F., Pamer, E. G., Littman, D. R. & Lang, R. A. In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens. *Immunity* (2002).
262. Bennett, C. L. & Clausen, B. E. DC ablation in mice: promises, pitfalls, and challenges. *Trends in Immunology* vol. 28 525–531 (2007).
263. Lu, T. X., Hartner, J., Lim, E., Fabry, V., Mingler, M. K., Manuscript, A., Mosser, D. M., Edwards, J. P. & Manuscript, A. The Dendritic Cell Lineage. *Changes* **41**, 15–25 (2009).
264. Joshi, S. K. & Lang, M. L. Fine tuning a well-oiled machine: Influence of NK1.1 and NKG2D on NKT cell development and function. *Int. Immunopharmacol.* **17**, 260–266 (2013).
265. Dashtsoodol, N., Shigeura, T., Ozawa, R., Harada, M., Kojo, S., Watanabe, T., Koseki, H., Nakayama, M., Ohara, O. & Taniguchi, M. Generation of novel Traj18-deficient mice lacking Vα14 natural killer T cells with an undisturbed T cell receptor α-chain repertoire. *PLoS One* **11**, e0153347 (2016).
266. Ismaili, J., Olislagers, V., Poupot, R., Fournié, J. J. & Goldman, M. Human γδ T cells induce dendritic cell maturation. *Clin. Immunol.* **103**, 296–302 (2002).
267. Koch, M., Stronge, V. S., Shepherd, D., Gadola, S. D., Mathew, B., Ritter, G., Fersht, A. R., Besra, G. S., Schmidt, R. R., Jones, E. Y. & Cerundolo, V. The crystal structure of human CD1d with and without α-galactosylceramide. *Nat. Immunol.* **6**, 819–826 (2005).
268. Moody, D. B., Zajonc, D. M. & Wilson, I. A. Anatomy of CD1-lipid antigen complexes. *Nature Reviews Immunology* vol. 5 387–399 (2005).
269. Koike, E., Takano, H., Inoue, K. ichiro, Yanagisawa, R. & Kobayashi, T. Carbon black nanoparticles promote the maturation and function of mouse bone marrow-derived dendritic cells. *Chemosphere* **73**, 371–376 (2008).
270. Zhu, R., Zhu, Y., Zhang, M., Xiao, Y., Du, X., Liu, H. & Wang, S. The induction of maturation on dendritic cells by TiO2 and Fe3O4@TiO2 nanoparticles via NF-κB signaling pathway. *Mater. Sci. Eng. C* **39**, 305–314 (2014).
271. Kang, K. & Lim, J.-S. Induction of Functional Changes of Dendritic Cells by Silica Nanoparticles. *Immune Netw.* **12**, 104 (2012).
272. Bedard, M., Shrestha, D., Priestman, D. A., Wang, Y., Schneider, F., Matute, J. D.,

Iyer, S. S., Gileadi, U., Prota, G., Kandasamy, M., Veerapen, N., Besra, G., Fritzsche, M., Zeissig, S., Shevchenko, A., Christianson, J. C., Platt, F. M., Eggeling, C., Blumberg, R. S. *et al.* Sterile activation of invariant natural killer T cells by ER-stressed antigen-presenting cells. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 23671–23681 (2019).

273. Christen, V. & Fent, K. Silica nanoparticles and silver-doped silica nanoparticles induce endoplasmatic reticulum stress response and alter cytochrome P4501A activity. *Chemosphere* **87**, 423–434 (2012).
274. Chen, R., Huo, L., Shi, X., Bai, R., Zhang, Z., Zhao, Y., Chang, Y. & Chen, C. Endoplasmic reticulum stress induced by zinc oxide nanoparticles is an earlier biomarker for nanotoxicological evaluation. *ACS Nano* **8**, 2562–2574 (2014).
275. Zhang, R., Piao, M. J., Kim, K. C., Kim, A. D., Choi, J. Y., Choi, J. & Hyun, J. W. Endoplasmic reticulum stress signaling is involved in silver nanoparticles-induced apoptosis. *Int. J. Biochem. Cell Biol.* **44**, 224–232 (2012).
276. Simard, J. C., Vallieres, F., De Liz, R., Lavastre, V. & Girard, D. Silver nanoparticles induce degradation of the endoplasmic reticulum stress sensor activating transcription factor-6 Leading to activation of the NLRP-3 Inflammasome. *J. Biol. Chem.* **290**, 5926–5939 (2015).
277. Belz, G. T., Smith, C. M., Kleinert, L., Reading, P., Brooks, A., Shortman, K., Carbone, F. R. & Heath, W. R. Distinct migrating and nonmigrating dendritic cell population are involved in MHC class I-restricted antigen presentation after lung infection with virus. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 8670–8675 (2004).
278. Lukens, M. V., Kruijsen, D., Coenjaerts, F. E. J., Kimpen, J. L. L. & van Bleek, G. M. Respiratory Syncytial Virus-Induced Activation and Migration of Respiratory Dendritic Cells and Subsequent Antigen Presentation in the Lung-Draining Lymph Node. *J. Virol.* **83**, 7235–7243 (2009).
279. Legge, K. L. & Braciale, T. J. Accelerated migration of respiratory dendritic cells to the regional lymph nodes is limited to the early phase of pulmonary infection. *Immunity* **18**, 265–277 (2003).
280. Misharin, A. V., Morales-Nebreda, L., Mutlu, G. M., Budinger, G. R. S. & Perlman, H. Flow cytometric analysis of macrophages and dendritic cell subsets in the mouse lung. *Am. J. Respir. Cell Mol. Biol.* **49**, 503–510 (2013).
281. Vignali, D. A. A. & Kuchroo, V. K. IL-12 family cytokines: immunological playmakers. *Nature Immunology* vol. 13 722–728 (2012).

282. Hochrein, H., Shortman, K., Vremec, D., Scott, B., Hertzog, P. & O'Keeffe, M. Differential Production of IL-12, IFN-α, and IFN-γ by Mouse Dendritic Cell Subsets. *J. Immunol.* **166**, 5448–5455 (2001).

283. Koch, F., Stanzl, U., Jennewein, P., Janke, K., Heufler, C., Kämpgen, E., Romani, N. & Schuler, G. High level IL-12 production by murine dendritic cells: Upregulation via MHC class II and CD40 molecules and downregulation by IL-4 and IL-10. *J. Exp. Med.* **184**, 741–746 (1996).

284. Pantel, A., Teixeira, A., Haddad, E., Wood, E. G., Steinman, R. M. & Longhi, M. P. Direct Type I IFN but Not MDA5/TLR3 Activation of Dendritic Cells Is Required for Maturation and Metabolic Shift to Glycolysis after Poly IC Stimulation. *PLoS Biol.* **12**, (2014).

285. Le Bon, A. & Tough, D. F. Links between innate and adaptive immunity via type I interferon. *Current Opinion in Immunology* (2002).

286. Blasius, A. L., Giurisato, E., Cella, M., Schreiber, R. D., Shaw, A. S. & Colonna, M. Bone Marrow Stromal Cell Antigen 2 Is a Specific Marker of Type I IFN-Producing Cells in the Naive Mouse, but a Promiscuous Cell Surface Antigen following IFN Stimulation. *J. Immunol.* **177**, 3260–3265 (2006).

287. Bollampalli, V. P., Harumi Yamashiro, L., Feng, X., Bierschenk, D., Gao, Y., Blom, H., Henriques-Normark, B., Nylén, S. & Rothfuchs, A. G. BCG Skin Infection Triggers IL-1R-MyD88-Dependent Migration of EpCAMlow CD11bhigh Skin Dendritic cells to Draining Lymph Node During CD4+ T-Cell Priming. *PLoS Pathog.* **11**, (2015).

288. Deckers, J., Sichien, D., Plantinga, M., Van Moorleghem, J., Vanheerswynghels, M., Hoste, E., Malissen, B., Dombrowicz, D., Guilliams, M., De Bosscher, K., Lambrecht, B. N. & Hammad, H. Epicutaneous sensitization to house dust mite allergen requires interferon regulatory factor 4–dependent dermal dendritic cells. *J. Allergy Clin. Immunol.* **140**, 1364-1377.e2 (2017).

289. Ochiai, S., Roediger, B., Abtin, A., Shklovskaya, E., Fazekas de St. Groth, B., Yamane, H., Weninger, W., Le Gros, G. & Ronchese, F. CD326 lo CD103 lo CD11b lo Dermal Dendritic Cells Are Activated by Thymic Stromal Lymphopoietin during Contact Sensitization in Mice . *J. Immunol.* **193**, 2504–2511 (2014).

290. Lee, H. K., Zamora, M., Linehan, M. M., Iijima, N., Gonzalez, D., Haberman, A. & Iwasaki, A. Differential roles of migratory and resident DCs in T cell priming

after mucosal or skin HSV-1 infection. *J. Exp. Med.* **206**, 359–370 (2009).

291. Hohl, T. M., Rivera, A., Lipuma, L., Gallegos, A., Shi, C., Mack, M. & Pamer, E. G. Inflammatory Monocytes Facilitate Adaptive CD4 T Cell Responses during Respiratory Fungal Infection. *Cell Host Microbe* **6**, 470–481 (2009).
292. Wüthrich, M., Ersland, K., Sullivan, T., Galles, K. & Klein, B. S. Fungi Subvert Vaccine T Cell Priming at the Respiratory Mucosa by Preventing Chemokine-Induced Influx of Inflammatory Monocytes. *Immunity* **36**, 680–692 (2012).
293. Peters, W., Cyster, J. G., Mack, M., Schlöndorff, D., Wolf, A. J., Ernst, J. D. & Charo, I. F. CCR2-Dependent Trafficking of F4/80 dim Macrophages and CD11c dim/intermediate Dendritic Cells Is Crucial for T Cell Recruitment to Lungs Infected with Mycobacterium tuberculosis . *J. Immunol.* **172**, 7647–7653 (2004).
294. Peters, W., Scott, H. M., Chambers, H. F., Flynn, J. L., Charo, I. F. & Ernst, J. D. Chemokine receptor 2 serves an early and essential role in resistance to Mycobacterium tuberculosis. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 7958–7963 (2001).
295. Chuang, Y.-H., Wang, T.-C., Jen, H.-Y., Yu, A. L. & Chiang, B.-L. α-Galactosylceramide–Induced Airway Eosinophilia Is Mediated through the Activation of NKT Cells. *J. Immunol.* **186**, 4687–4692 (2011).
296. Kelly, J., Minoda, Y., Meredith, T., Cameron, G., Philipp, M. S., Pellicci, D. G., Corbett, A. J., Kurts, C., Gray, D. H. D., Godfrey, D. I., Kannourakis, G. & Berzins, S. P. Chronically stimulated human MAIT cells are unexpectedly potent IL-13 producers. *Immunol. Cell Biol.* **97**, 689–699 (2019).
297. Segal, A. W. How Neutrophils Kill Microbes. *Annu. Rev. Immunol.* **23**, 197–223 (2005).
298. Wingender, G., Hiss, M., Engel, I., Peukert, K., Ley, K., Haller, H., Kronenberg, M. & von Vietinghoff, S. Neutrophilic Granulocytes Modulate Invariant NKT Cell Function in Mice and Humans. *J. Immunol.* **188**, 3000–3008 (2012).
299. Schneider, M., Hannaway, R. F., Lamichhane, R., de la Harpe, S. M., Tyndall, J. D. A., Vernall, A. J., Kettle, A. J. & Ussher, J. E. Neutrophils suppress mucosal-associated invariant T cells in humans. *Eur. J. Immunol.* **50**, 643–655 (2020).
300. Jones, H. R., Robb, C. T., Perretti, M. & Rossi, A. G. The role of neutrophils in inflammation resolution. *Seminars in Immunology* vol. 28 137–145 (2016).
301. Laan, M., Cui, Z. H., Hoshino, H., Lötvall, J., Sjöstrand, M., Gruenert, D. C., Skoogh, B. E. & Lindén, A. Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. *J. Immunol.* **162**, 2347–52 (1999).

302. Jones, C. E. & Chan, K. Interleukin-17 stimulates the expression of interleukin-8, growth-related oncogene-α, and granulocyte-colony-stimulating factor by human airway epithelial cells. *Am. J. Respir. Cell Mol. Biol.* **26**, 748–753 (2002).

303. Fossiez, F., Djossou, O., Chomarat, P., Flores-Romo, L., Ait-Yahia, S., Maat, C., Pin, J. J., Garrone, P., Garcia, E., Saeland, S., Blanchard, D., Gaillard, C., Das Mahapatra, B., Rouvier, E., Golstein, P., Banchereau, J. & Lebecque, S. T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. *J. Exp. Med.* **183**, 2593–2603 (1996).

304. Ling, P., Gately, M. K., Gubler, U., Stern, A. S., Lin, P., Hollfelder, K., Su, C., Pan, Y. C. & Hakimi, J. Human IL-12 p40 homodimer binds to the IL-12 receptor but does not mediate biologic activity. *J. Immunol.* **154**, 116–27 (1995).

305. Gillessen, S., Carvajal, D., Ling, P., Podlaski, F. J., Stremlo, D. L., Familletti, P. C., Gubler, U., Presky, D. H., Stern, A. S. & Gately, M. K. Mouse interleukin-12 (IL-12) p40 homodimer: a potent IL-12 antagonist. *Eur. J. Immunol.* **25**, 200–206 (1995).

306. Marks, E., Naudin, C., Nolan, G., Goggins, B. J., Burns, G., Mateer, S. W., Latimore, J. K., Minahan, K., Plank, M., Foster, P. S., Callister, R., Veysey, M., Walker, M. M., Talley, N. J., Radford-Smith, G. & Keely, S. Regulation of IL-12p40 by HIF controls Th1/Th17 responses to prevent mucosal inflammation. *Mucosal Immunol.* **10**, 1224–1236 (2017).

307. Lee, S.-Y., Jung, Y. O., Kim, D.-J., Kang, C.-M., Moon, Y.-M., Heo, Y.-J., Oh, H.-J., Park, S.-J., Yang, S.-H., Kwok, S. K., Ju, J.-H., Park, S.-H., Sung, Y. C., Kim, H.-Y. & Cho, M.-L. IL-12p40 Homodimer Ameliorates Experimental Autoimmune Arthritis. *J. Immunol.* **195**, 3001–3010 (2015).

308. Kim, D. J., Kim, K. S., Song, M. Y., Seo, S. H., Kim, S. J., Yang, B. G., Jang, M. H. & Sung, Y. C. Delivery of IL-12p40 ameliorates DSS-induced colitis by suppressing IL-17A expression and inflammation in the intestinal mucosa. *Clin. Immunol.* **144**, 190–199 (2012).

309. Komai-Koma, M., Jones, L., Ogg, G. S., Xu, D. & Liew, F. Y. TLR2 is expressed activated T cells as a costimulatory receptor. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 3029–3034 (2004).

310. Muzio, M., Natoli, G., Saccani, S., Levrero, M. & Mantovani, A. The human toll signaling pathway: Divergence of nuclear factor κb and jnk/sapk activation upstream of tumor necrosis factor receptor-associated factor 6 (TRAF6). *J. Exp.*

Med. **187**, 2097–2101 (1998).

311. Askenase, P. W., Itakura, A., Leite-de-Moraes, M. C., Lisbonne, M., Roongapinun, S., Goldstein, D. R. & Szczepanik, M. TLR-Dependent IL-4 Production by Invariant Vα14 + Jα18 + NKT Cells to Initiate Contact Sensitivity In Vivo . *J. Immunol.* **175**, 6390–6401 (2005).
312. Campos, R. A., Szczepanik, M., Itakura, A., Lisbonne, M., Dey, N., Leite-De-Moraes, M. C. & Askenase, P. W. Interleukin-4-dependent innate collaboration between iNKT cells and B-1 B cells controls adaptative contact sensitivity. *Immunology* **117**, 536–547 (2006).
313. Campos, R. A., Szczepanik, M., Itakura, A., Akahira-Azuma, M., Sidobre, S., Kronenberg, M. & Askenase, P. W. Cutaneous Immunization Rapidly Activates Liver Invariant Vα14 NKT Cells Stimulating B-1 B Cells to Initiate T Cell Recruitment for Elicitation of Contact Sensitivity. *J. Exp. Med.* **198**, 1785–1796 (2003).
314. Nagarajan, N. A. & Kronenberg, M. Invariant NKT Cells Amplify the Innate Immune Response to Lipopolysaccharide. *J. Immunol.* **178**, 2706–2713 (2007).
315. Godfrey, D. I., Pellicci, D. G., Patel, O., Kjer-Nielsen, L., McCluskey, J. & Rossjohn, J. Antigen recognition by CD1d-restricted NKT T cell receptors. *Semin. Immunol.* **22**, 61–67 (2010).
316. Pellicci, D. G., Patel, O., Kjer-Nielsen, L., Pang, S. S., Sullivan, L. C., Kyparissoudis, K., Brooks, A. G., Reid, H. H., Gras, S., Lucet, I. S., Koh, R., Smyth, M. J., Mallevaey, T., Matsuda, J. L., Gapin, L., McCluskey, J., Godfrey, D. I. & Rossjohn, J. Differential Recognition of CD1d-α-Galactosyl Ceramide by the Vβ8.2 and Vβ7 Semi-invariant NKT T Cell Receptors. *Immunity* **31**, 47–59 (2009).
317. Zajonc, D. M., Cantu, C., Mattner, J., Zhou, D., Savage, P. B., Bendelac, A., Wilson, I. A. & Teyton, L. Structure and function of a potent agonist for the semi-invariant natural killer T cell receptor. *Nat. Immunol.* **6**, 810–818 (2005).
318. Grabowski, G. A., Barton, N. W., Pastores, G., Dambrosia, J. M., Banerjee, T. K., McKee, M. A., Parker, C., Schiffmann, R., Hill, S. C. & Brady, R. O. Enzyme therapy in type 1 Gaucher disease: Comparative efficacy of mannose- terminated glucocerebrosidase from natural and recombinant sources. *Ann. Intern. Med.* **122**, 33–39 (1995).
319. Weinreb, N. J. Imiglucerase and its use for the treatment of Gaucher's disease.

Expert Opin. Pharmacother. **9**, 1987–2000 (2008).

320. Grommé, M., Uytdehaag, F. G. C. M., Janssen, H., Calafat, J., Van Binnendijk, R. S., Kenter, M. J. H., Tulp, A., Verwoerd, D. & Neefjes, J. Recycling MHC class I molecules and endosomal peptide loading. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 10326–10331 (1999).
321. Compeer, E. B., Flinsenberg, T. W. H., van der Grein, S. G. & Boes, M. Antigen processing and remodeling of the endosomal pathway: Requirements for antigen cross-presentation. *Front. Immunol.* **3**, 1–11 (2012).
322. Burgdorf, S., Schölz, C., Kautz, A., Tampé, R. & Kurts, C. Spatial and mechanistic separation of cross-presentation and endogenous antigen presentation. *Nat. Immunol.* **9**, 558–566 (2008).
323. Rahman, A. H., Taylor, D. K. & Turka, L. A. The contribution of direct TLR signaling to T cell responses. *Immunologic Research* vol. 45 25–36 (2009).
324. Meierovics, A., Yankelevich, W. J. C. & Cowley, S. C. MAIT cells are critical for optimal mucosal immune responses during in vivo pulmonary bacterial infection. *Proc. Natl. Acad. Sci. U. S. A.* **110**, E3119-28 (2013).
325. Decrausaz, L., Gonçalves, A. R., Domingos-Pereira, S., Pythoud, C., Stehle, J. C., Schiller, J., Jichlinski, P. & Nardelli-Haefliger, D. A novel mucosal orthotopic murine model of human papillomavirus-associated genital cancers. *Int. J. Cancer* **128**, 2105–2113 (2011).
326. Wang, R. F., Appella, E., Kawakami, Y., Kang, X. & Rosenberg, S. A. Identification of TRP-2 as a human tumor antigen recognized by cytotoxic T lymphocytes. *J. Exp. Med.* **184**, 2207–2216 (1996).
327. Xu, Z., Ramishetti, S., Tseng, Y. C., Guo, S., Wang, Y. & Huang, L. Multifunctional nanoparticles co-delivering Trp2 peptide and CpG adjuvant induce potent cytotoxic T-lymphocyte response against melanoma and its lung metastasis. *J. Control. Release* **172**, 259–265 (2013).
328. Kawakami, Y., Eliyahu, S., Delgado, C. H., Robbins, P. F., Sakaguchi, K., Appella, E., Yannelli, J. R., Adema, G. J., Miki, T. & Rosenberg, S. A. Identification of a human melanoma antigen recognized by tumor- infiltrating lymphocytes associated with in vivo tumor rejection. *Proc. Natl. Acad. Sci. U. S. A.* **91**, 6458–6462 (1994).
329. Rosenberg, S. A., Yang, J. C., Schwartzentruber, D. J., Hwu, P., Marincola, F. M., Topalian, S. L., Restifo, N. P., Dudley, M. E., Schwarz, S. L., Spiess, P. J.,

Wunderlich, J. R., Parkhurst, M. R., Kawakami, Y., Seipp, C. A., Einhorn, J. H. & White, D. E. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. *Nat. Med.* **4**, 321–327 (1998).

330. Tuettenberg, A., Becker, C., Huter, E., Knop, J., Enk, A. H. & Jonuleit, H. Induction of strong and persistent MelanA/MART-1-specific immune responses by adjuvant dendritic cell-based vaccination of stage II melanoma patients. *Int. J. Cancer* **118**, 2617–2627 (2006).
331. Gallichan, W. S., Woolstencroft, R. N., Guarasci, T., McCluskie, M. J., Davis, H. L. & Rosenthal, K. L. Intranasal Immunization with CpG Oligodeoxynucleotides as an Adjuvant Dramatically Increases IgA and Protection Against Herpes Simplex Virus-2 in the Genital Tract. *J. Immunol.* **166**, 3451–3457 (2001).
332. Perrone, L. A., Ahmad, A., Veguilla, V., Lu, X., Smith, G., Katz, J. M., Pushko, P. & Tumpey, T. M. Intranasal Vaccination with 1918 Influenza Virus-Like Particles Protects Mice and Ferrets from Lethal 1918 and H5N1 Influenza Virus Challenge. *J. Virol.* **83**, 5726–5734 (2009).
333. Forbes, E. K., Sander, C., Ronan, E. O., McShane, H., Hill, A. V. S., Beverley, P. C. L. & Tchilian, E. Z. Multifunctional, High-Level Cytokine-Producing Th1 Cells in the Lung, but Not Spleen, Correlate with Protection against Mycobacterium tuberculosis Aerosol Challenge in Mice . *J. Immunol.* **181**, 4955–4964 (2008).
334. Tomusange, K., Wijesundara, D., Gummow, J., Wesselingh, S., Suhrbier, A., Gowans, E. J. & Grubor-Bauk, B. Mucosal vaccination with a live recombinant rhinovirus followed by intradermal DNA administration elicits potent and protective HIV-specific immune responses. *Sci. Rep.* **6**, (2016).
335. Belyakov, I. M. & Berzofsky, J. A. Immunobiology of mucosal HIV infection and the basis for development of a new generation of mucosal AIDS vaccines. *Immunity* vol. 20 247–253 (2004).
336. Haynes, B. F., Moody, M. A., Alam, M., Bonsignori, M., Verkoczy, L., Ferrari, G., Gao, F., Tomaras, G. D., Liao, H. X. & Kelsoe, G. Progress in HIV-1 vaccine development. *Journal of Allergy and Clinical Immunology* vol. 134 3–10 (2014).
337. Sandoval, F., Terme, M., Nizard, M., Badoual, C., Bureau, M. F., Freyburger, L., Clement, O., Marcheteau, E., Gey, A., Fraisse, G., Bouguin, C., Merillon, N., Dransart, E., Tran, T., Quintin-Colonna, F., Autret, G., Thiebaud, M., Suleman,

M., Riffault, S. *et al.* Mucosal imprinting of vaccine-induced CD8+ T cells is crucial to inhibit the growth of mucosal tumors. *Sci. Transl. Med.* **5**, (2013).

338. Wakabayashi, A., Nakagawa, Y., Shimizu, M., Moriya, K., Nishiyama, Y. & Takahashi, H. Suppression of an Already Established Tumor Growing through Activated Mucosal CTLs Induced by Oral Administration of Tumor Antigen with Cholera Toxin. *J. Immunol.* **180**, 4000–4010 (2008).
339. Mullins, D. W., Sheasley, S. L., Ream, R. M., Bullock, T. N. J., Fu, Y. X. & Engelhard, V. H. Route of immunization with peptide-pulsed dendritic cells controls the distribution of memory and effector T cells in lymphoid tissues and determines the pattern of regional tumor control. *J. Exp. Med.* **198**, 1023–1034 (2003).
340. Kim-Schulze, S., Kim, H. S., Wainstein, A., Kim, D. W., Yang, W. C., Moroziewicz, D., Mong, P. Y., Bereta, M., Taback, B., Wang, Q. & Kaufman, H. L. Intrarectal Vaccination with Recombinant Vaccinia Virus Expressing Carcinoembronic Antigen Induces Mucosal and Systemic Immunity and Prevents Progression of Colorectal Cancer. *J. Immunol.* **181**, 8112–8119 (2008).
341. Lacharme-Lora, L., Salisbury, V., Humphrey, T. J., Stafford, K. & Perkins, S. E. Bacteria isolated from parasitic nematodes - A potential novel vector of pathogens? in *Environmental Health: A Global Access Science Source* (2009).
342. Anderson, G. L., Kenney, S. J., Millner, P. D., Beuchat, L. R. & Williams, P. L. Shedding of foodborne pathogens by Caenorhabditis elegans in compost-amended and unamended soil. *Food Microbiol.* (2006).
343. Kenney, S. J., Anderson, G. L., Williams, P. L., Millner, P. D. & Beuchat, L. R. Persistence of Escherichia coli O157:H7, Salmonella Newport, and Salmonella Poona in the gut of a free-living nematode, Caenorhabditis elegans, and transmission to progeny and uninfected nematodes. *Int. J. Food Microbiol.* (2005).
344. Klion, A. D. & Nutman, T. B. The role of eosinophils in host defense against helminth parasites. *J. Allergy Clin. Immunol.* **113**, 30–37 (2004).
345. Chen, F., Liu, Z., Wu, W., Rozo, C., Bowdridge, S., Millman, A., Van Rooijen, N., Urban, J. F., Wynn, T. A. & Gause, W. C. An essential role for T H 2-type responses in limiting acute tissue damage during experimental helminth infection. *Nat. Med.* **18**, 260–266 (2012).
346. Chen, F., Wu, W., Millman, A., Craft, J. F., Chen, E., Patel, N., Boucher, J. L., Urban, J. F., Kim, C. C. & Gause, W. C. Neutrophils prime a long-lived effector

macrophage phenotype that mediates accelerated helminth expulsion. *Nat. Immunol.* **15**, 938–946 (2014).

347. Anthony, R. M., Rutitzky, L. I., Urban, J. F., Stadecker, M. J. & Gause, W. C. Protective immune mechanisms in helminth infection. *Nature Reviews Immunology* vol. 7 975–987 (2007).
348. Humbert, M., Durham, S. R., Ying, S., Kimmitt, P., Barkans, J., Assoufi, B., Pfister, R., Menz, G., Robinson, D. S., Kay, A. B. & Corrigan, C. J. IL-4 and IL-5 mRNA and protein in bronchial biopsies from patients with atopic and nonatopic asthma: Evidence against 'intrinsic' asthma being a distinct immunopathologic entity. *Am. J. Respir. Crit. Care Med.* **154**, 1497–1504 (1996).
349. Chandra, S., Wingender, G., Greenbaum, J. A., Khurana, A., Gholami, A. M., Ganesan, A.-P., Rosenbach, M., Jaffee, K., Gern, J. E., Wood, R., O'Connor, G., Sandel, M., Kattan, M., Bacharier, L., Togias, A., Horner, A. A. & Kronenberg, M. Development of Asthma in Inner-City Children: Possible Roles of MAIT Cells and Variation in the Home Environment. *J. Immunol.* **200**, 1995–2003 (2018).
350. Lezmi, G., Abou Taam, R., Dietrich, C., Chatenoud, L., de Blic, J. & Leite-de-Moraes, M. Circulating IL-17-producing mucosal-associated invariant T cells (MAIT) are associated with symptoms in children with asthma. *Clin. Immunol.* **188**, 7–11 (2018).
351. Camberis, M., Le Gros, G. & Urban, J. Animal Model of Nippostrongylus brasiliensis and Heligmosomoides polygyrus . *Current Protocols in Immunology* (2003).
352. Nussbaum, J. C., Van Dyken, S. J., Von Moltke, J., Cheng, L. E., Mohapatra, A., Molofsky, A. B., Thornton, E. E., Krummel, M. F., Chawla, A., Liang, H. E. & Locksley, R. M. Type 2 innate lymphoid cells control eosinophil homeostasis. *Nature* (2013).
353. Huang, Y., Guo, L., Qiu, J., Chen, X., Hu-Li, J., Siebenlist, U., Williamson, P. R., Urban, J. F. & Paul, W. E. IL-25-responsive, lineage-negative KLRG1 hi cells are multipotential 'inflammatory' type 2 innate lymphoid cells. *Nat. Immunol.* **16**, 161–169 (2015).